Identification and Characterisation of *Arabidopsis* Systemic Acquired Resistance Mutants isolated by Luciferase Imaging

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Contents

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Abstract

Plants have evolved a complex series of integrated defence mechanisms against pathogens. Following recognition of a pathogen avirulence (*avr*) gene product by the corresponding plant resistance (*R*) gene product, a complex signalling network is initiated. Local inducible defences are activated and a long-distance signal is released, leading to the establishment of systemic acquired resistance (SAR) to a wide range of pathogens. SAR is marked by the accumulation of pathogenesis-related (PR) proteins. Salicylic acid (SA) is a key signalling molecule in SAR, inducing *PR* gene expression both locally and systemically. However, it is thought that signal transduction leading to acquired disease resistance differs for biotrophic and necrotrophic pathogens. Generally, SA is required for resistance to biotrophic pathogens only. SA-independent resistance to necrotrophic pathogens is associated with jasmonic acid (JA) and ethylene signalling and is marked by the production of antimicrobial proteins.

In order to study further the molecular basis of SAR, we have developed a method of identifying novel SAR mutants by luciferase imaging. Transgenic *Arabidopsis thaliana* plants expressing a *PR-1a:*luciferase reporter gene were generated and homozygous seed was chemically mutagenised. Mutants with perturbations in *PR-1* gene expression were identified and could be divided into various classes. A novel mutant expressing *PR-1* constitutively was selected for further study. *cir1* (*c*onstitutively *i*nduced *r*esistance *1*) expressed both SA-dependent and SA-independent defence genes constitutively, accumulated SA to high levels and produced an increased amount of ethylene. In addition, *cir1* exhibited resistance to the virulent bacterial pathogen *Pseudomonas syringae* pv *tomato* DC3000 and the virulent oomycete pathogen *Peronospora parasitica* Noco2. Genetic analyses indicated that *cir1* is recessive and defines a mutation in a single gene. *cir1* mapped to the lower arm of chromosome 4. Double mutants were produced between *cir1* and SA-, JA- and ethylene-insensitive mutants. Analysis of these plants showed that SA, JA and ethylene were required for constitutive defence gene expression and disease resistance in *cir1*. Thus, the results obtained indicate that CIR1 acts as a negative regulator in the disease resistance signal transduction network, most likely functioning upstream of the branchpoint between the SA-dependent and SA-independent pathways.

Abbreviations

bp	basepairs
ceb	constitutive expression of bioluminescence
cfu	colony forming units
cir1	constitutively induced resistance 1
Col-0	*Arabidopsis* ecotype Columbia
heb	high expression of bioluminescence
ISR	induced systemic resistance
JA	jasmonic acid
kb	kilobase
KB	King's broth media
L*er*	*Arabidopsis* ecotype Landsberg *erecta*
luc	luciferase
mRNA	messenger ribonucleic acid
Me-JA	methyl jasmonate
MS	Murashige and Skoog media
ng	nanagram
neb	no expression of bioluminescence
nt	nucleotide
Psm	*Pseudomonas syringae* pv *maculicola*
Pst	*Pseudomonas syringae* pv *tomato*
PCR	polymerase chain reaction
RE	restriction enzyme
RLU	relative light units
rpm	revolutions per minute
SA	salicylic acid
SAR	systemic acquired resistance
UK4	*Arabidopsis* ecotype Umkirch 4
U	unit
Ws	*Arabidopsis* ecotype Wassilewskija

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Chapter One
General Introduction

1. 1. Introduction

It has been estimated that 12% of potential global crop production is lost annually to pre-harvest plant disease (Agrios 1997, Shah 1997). Outbreaks of disease are a result of interactions between cultivated crops and pathogens (bacteria, fungi, insects, viruses and nematodes). Despite the development and use of an increasing number of pesticides and fungicides, crop losses due to disease still continue. Plant breeding has been used to introduce resistance genes from wild populations into commercial crop cultivars, but this resistance often is not durable as pathogens are able to evolve quickly and overcome it (Agrios 1997). Plant transformation technology has been developed over the last decade for several commercially important crops and offers exciting opportunities for engineering crop protection (Shah 1997). Advances are also being made in the understanding of plant-pathogen interactions. These include the isolation of a number of resistance (*R*) genes and the analysis of signalling pathways leading to the hypersensitive response (HR) and systemic acquired resistance (SAR) (Hammerschmidt 1999, Martin 1999, Malek & Lawton 1998, Bent 1996, Hammond-Kosack & Jones 1996, Jackson et al. 1996, Ryals et al. 1996). Thus, further understanding of these plant disease resistance processes may pave the way for the development of transgenic crops with increased disease resistance, or novel pesticides capable of activating plant defence responses.

1.2. Disease resistance in plants

Types of disease resistance

Plants have the potential to respond to pathogen attack by employing one of two broad strategies. Firstly, structural and pre-formed chemical barriers such as saponins prevent the pathogen from gaining sustenance from the host (Hammerschmidt 1999, Glazebrook et al.1997a, Osbourn 1996). Secondly, the plant can engage a number of defence mechanisms aimed at limiting pathogen spread

(Hammerschmidt 1999, Glazebrook et al.1997a, Hammond-Kosack & Jones 1996). These defence mechanisms include the hypersensitive response (HR), the production of reactive oxygen species (ROS), fortification of the cell wall by lignin polymerization and expression of a number of genes including those encoding pathogenesis-related (PR) proteins, thionins, defensins, glutathione-S-transferases, phenylalanine ammonia lyase (PAL) and enzymes involved in phytoalexin biosynthesis (Glazebrook et al.1997a, Hammond-Kosack & Jones 1996). Gene-for-gene resistance is said to occur when the plant is capable of rapidly engaging these defence responses.

The use of Arabidopsis thaliana in understanding disease resistance

Arabidopsis thaliana (L.) Heynh. (Arabidopsis), a small diploid plant in the Brassicaceae family, has been developed as a model genetic system for research in plant science (Meinke et al. 1998). The reasons for this choice are many. Arabidopsis is easy to grow both in tissue culture and in the green house, and many plants can be grown in a relatively small area. The entire life cycle, including seed germination, formation of a rosette plant, bolting of the main stem, flowering, sillique formation and maturation of the first seeds can be completed in approximately two months (Meinke et al. 1998). Many methods for Arabidopsis research have been developed, including chemical and insertional mutagenesis, efficient transformation methods and an extensive collection of mutants with a diverse range of phenotypes (Clough & Bent 1998, Koncz et al. 1992, http://www.arabidopsis.org/). The 120-megabase Arabidopsis genome is organised into five chromosomes and contains an estimated 27 000 genes (Meinke et al. 1998). Three related constantly evolving maps of each chromosome (classical genetic, recombinant inbred and physical maps; Lister & Dean 1993, Rhee et al. 1999) containing a number of different markers are deposited at The Arabidopsis Information Resource (TAIR) (http://www.arabidopsis.org/). A consortium of laboratories is in the process of sequencing the entire genome with the published sequence expected in December 2000, and the complete annotated sequence of chromosome 2 and 4 has already been deposited into Genbank (Lin et al. 1999, Mayer et al. 1999).

In addition to the genetic advantages of using Arabidopsis as a model system, it is a very good system for studying host defence responses to pathogen attack. A large number of virulent and avirulent bacterial, fungal and viral pathogens have been identified and used to identify many disease resistance mutants (Glazebrook et al.1997a, Kunkel 1996). On-going analysis of these mutants is proving to be a very important tool for the dissection of signal transduction networks leading to induction of defence responses (Glazebrook et al.1997a, Kunkel 1996).

1.3. Gene-for-Gene resistance

If during interaction with a plant, a pathogen is able to penetrate the plant and cause disease, the pathogen is said to be virulent, the plant susceptible and the interaction compatible. On the other hand, the plant may be able to activate defence responses more quickly, thus preventing the development of disease. In this case, the pathogen is said to be avirulent, the plant resistant and the interaction incompatible. In the gene-for-gene model for plant disease resistance, first proposed by Flor, an incompatible interaction has been hypothesised to result from the interaction of the product of a plant resistance (*R*) gene with the product of the corresponding avirulence (*avr*) gene (Flor 1971, Keen 1992). *R-avr* interactions have been observed between plants and many different pathogens including bacteria, fungi, viruses and nematodes, and in general, a specific *R* gene product will interact with only the corresponding *avr* product (Crute & Pink 1996, Keen 1992). A simple explanation of this model is that *avr* genes encode ligands that bind to receptors encoded by the plant *R* genes (Glazebrook et al.1997a). Binding of the ligand to the receptor then stimulates a complex signal transduction cascade that may involve protein phosphorylation, ion fluxes, ROS and activation of transcription factors, culminating in the expression of defence response genes and resistance (Yang et al. 1997, Hammond-Kosack & Jones 1996). In the case of resistance to virulent pathogens, no HR is visualised and defence responses are activated more slowly (Glazebrook et al. 1997a).

Avr genes

To date, over 40 *avr* genes have been isolated from bacterial and fungal pathogens (Gabriel 1999). In addition to *avr* genes, avirulent bacterial pathogens possess hypersensitive response and pathogenicity (*hrp*) genes, which have been shown to be necessary for an avirulent bacteria to elicit an HR in a resistant plant (Collmer 1998, Lindgren 1997). The *hrp* genes encode a type III protein-secretion system that appears to be capable of delivering the avr protein across the bacterial and plant cell walls and into the plant cell, where it can interact with the corresponding R protein (Collmer 1998, Lindgren 1997). On the other hand, cloned fungal *avr* genes have been predicted or shown to encode extracellular proteins (Lauge & DeWit 1998, Knogge 1996). Elicitors such as oligogalactoronates are also released from plant cells following attack by a number of different fungi, resulting in plant defence responses (Knogge 1996). Although the biological function of avr is unknown, it is possible that *avr* genes encode pathogenicity factors (Gabriel 1999). Recently *virPphA*, the first virulence (*vir*) gene described from the soybean bacterial pathogen *Pseudomonas syringae* pv *phaseolicola* (Pph), was isolated (Jackson et al. 1999). Pph strains cured of a 154-kb plasmid lost virulence towards previously susceptible cultivars of soybean. Restoration of virulence was achieved by complementation with a cosmid clone containing a 30-kb region of the plasmid, which had previously been shown to contain three *avr* genes (Jackson et al. 1999). Sequencing of this clone revealed three putative *vir* genes that were predicted to encode hydrophilic proteins. One gene, designated *virPphA*, achieved partial resoration of virulence when cloned on it's own, and also acted as an *avr* gene in some soybean cultivars, rapidly inducing the HR (Jackson et al. 1999). This example illustrates that a bacterial virulence factor acts as an avr protein, presumably when it interacts with the corresponding plant *R* gene product. In addition, it seems likely that the presence of an *avr* gene would give the pathogen an advantage during a compatible interaction. In fungal pathogens, it has been proposed that avr proteins are required for fungal fitness in the field (Knogge 1996).

R genes

A number of *R* genes have been isolated from tomato, Arabidopsis, tobacco, flax, maize, barley, rice and potato (reviewed in Buell 1998, Baker et al. 1997, Bent 1996, Hammond-Kosack & Jones 1996). The majority of *R* genes contain a leucine-rich repeat domain (LRR), which implies a role in protein-protein interactions (Bent 1996). Additional sequence features present in some *R* genes include a signal sequence, nucleotide binding site (NBS), leucine zipper (LZ), transmembrane domain, glycosolation sites, a kinase domain and Toll-IL-IR homology region (TIR) (Buell 1998, Baker et al. 1997, Bent 1996). TIRs have homology to the *Drosophila* developmental gene *Toll* and the mammalian immune response gene encoding the interleukin-1 receptor (IL-IR), both of which play a role in defence (Baker et al.1997). *R* genes cloned to date can be divided into five classes, which can be defined as follows: a detoxification enzyme which is activated in a gene-for-gene manner, an intracellular protein kinase, an intracellular LRR-NBS class (which can be sub-divided into LZ/NBS/LRR and TIR/NBS/LRR proteins), an extracellular LRR protein with a single membrane spanning region and short cytoplasmic carboxyl terminus, and an extracellular LRR protein with a cytoplasmic kinase domain (reviewed in Hammond-Kosack & Jones 1997). These five classes of *R* genes, with examples of each, are outlined in Table 1.1. The barley *Mlo* resistance gene to powdery mildew falls outside of this classification, as it encodes a novel protein with six membrane-spanning helices, and differs from other *R* genes in that it is recessive and confers resistance in a non-race specific manner (Buschages et al. 1997).

Table1.1. The five classes of cloned *R* genes (adapted from Hammond-Kosack & Jones 1997).

Class	R protein predicted features	Gene	Plant	Pathogen	Pathogen type	Reference
1	Detoxifying enzyme	*Hm1*	Maize	*Helminthosporum maydis* (race 1)	Necrotrophic fungus	Johal et al. 1992
2	Intracellular protein kinase	*Pto*	Tomato	*P. syringae pv tomato (avrPto)*	Extracellular bacteria	Martin et al. 1993
3	NBS/LRR	*Xa1*	Rice	*Xanthomonas oryzae* pv *oryzae* (race1)	Extracellular bacteria	Yoshimura et al. 1998
3a	LZ/NBS/LRR	*RPS2*	Arabidopsis	*P.syringae pv tomato (avrRpt2)*	Extracellular bacteria	Mindrinos et al. 1994
		RPM1	Arabidopsis	*P.syringae pv maculicola (avrRpm1/avrB)*	Extracellular bacteria	Grant et al. 1995
		RPP8	Arabidopsis	*Peronospora parasitica*	Biotrophic downy mildew oomycete	McDowell et al. 1998
		RPS5	Arabidopsis	*P. syringae* pv *tomato (avrPphB)*	Extracellular bacteria	Warren et al.1998
		Mi	Tomato	*Meloidogyne javanica* and *Macrosiphum euphorbiae*	Root-knot nematode and potato aphid	Milligan et 1998, Rossi et al. 1998
		RPP13	Arabidopsis	*P.parasitica*	Downy mildew	Bittner-Eddy et al. 2000
		HRT	Arabidopsis	Turnip crinkle virus	Intracellular virus	Cooley et al. 2000
3b	TIR/NBS/LRR	*N*	Tobacco	Tobacco mosaic virus	Intracellular virus	Whitham et al. 1994
		L6	Flax	*Melamspora lini (AL6)*	Biotrophic fungal rust	Lawrence et al. 1995
		RPP5	Arabidopsis	*Peronospora parasitica*	Downy mildew	Parker et al. 1997
		RPP1 genes	Arabidopsis	*P.parasitica*	Downy mildew	Botella et al. 1998
		RPS4	Arabidopsis	*P.syringae pv tomato (avrRps4)*	Extracellular bacteria	Gassman et al. 1999
4	Extracellular LRR with single membrane spanning region and short cytoplasmic carboxyl terminus	*Cf-9, Cf-2, Cf-4, Cf-5*	Tomato	*Cladosporium fulvum (Avr9, Avr2, Avr4, Avr5)*	Biotrophic extracellular fungus	Jones et al. 1994, Dixon et al. 1996, Hammond-Kosack & Jones 1997
5	Extracellular LRR with single memebrane spanning region and cytoplasmic kinase domain.	*Xa-21*	Rice	*X. oryzae pv oryzae* (all races)	Extracellular bacteria	Song et al. 1995

To date, over 49 pathogen specificity loci in Arabidopsis for various pathogens have been identified and mapped (Buell 1998). These include genes conferring disease resistance to the bacterial pathogens *Pseudomonas syringae* and *Xanthomonas campestris*, the oomycete pathogens *Peronospora parasitica* and *Albugo candida,* the powdery mildew fungal pathogens *Erisiphe cichoracearum, Erisiphe cruciferarum* and *Erisiphe orontii*, the clubroot pathogen *Plasmodiophora brassicae,* and the viral pathogens tobacco ringspot virus, turnip crinkle virus, cauliflower mosaic virus and beet curly top virus (reviewed in Buell 1998, Glazebrook et al.1997a). Some of these Arabidopsis *R* genes have been cloned using map based strategies and these are included in Table 1.1. Of interest to the present study is the interaction between the products of the *RPM1* and *avrB* genes. It was previously established that *avrB* corresponded to the *R* gene *RPS3*, but later it was shown that *RPM1* and *RPS3* were the same gene by complementation (Bisgrove et al. 1994). Thus the *RPM1* gene represents a deviation from the gene-for-gene theory as the RPM1 protein can bind one of two avr proteins: the products of the *avrB* and *avrRPM1* genes (Glazebrook et al. 1997a). The RPM1 protein has been localised by epitope tagging, and is a peripheral membrane protein most likely residing on the cytoplasmic face of the plasmamembrane (Boyes et al. 1998).

1.4. Acquired resistance

Systemic Acquired Resistance

Systemic acquired resistance (SAR) is an inducible defence response that leads to broad spectrum, long-lasting systemic resistance following inoculation with an 'immunising' pathogen (Delaney 1997, Sticher et al. 1997, Ryals et al. 1996, Ryals et al. 1994, Ross 1961). Systemic resistance is initiated in addition to locally induced defence responses. SAR is initiated when a resistant plant is inoculated with an avirulent pathogen, leading to the formation of the HR and localised necrosis (Fig.1.1). SAR can be expressed in both monocots and dicots against a broad range of pathogens, which may differ from the SAR-inducing organism (Sticher et al. 1997). SAR has been studied extensively in tobacco, cucumber and more recently Arabidopsis, and it has been established that the time needed for the establishment of

SAR depends on both the plant and the type of pathogen. In addition, concentration of the pathogen used in inoculation influences the level of protection (Sticher et al. 1997). It has been proposed that SAR is induced by translocation of an unknown signal from the site of primary inoculation to the rest of the plant (Fig.1.1). This signal then primes the plant for subsequent attack by the secondary, virulent pathogen, most probably by triggering a complex array of defence responses (Fig.1.1), which include the expression of pathogenesis-related proteins and antimicrobial peptides (Ryals et al. 1994, Penninckx et al. 1996).

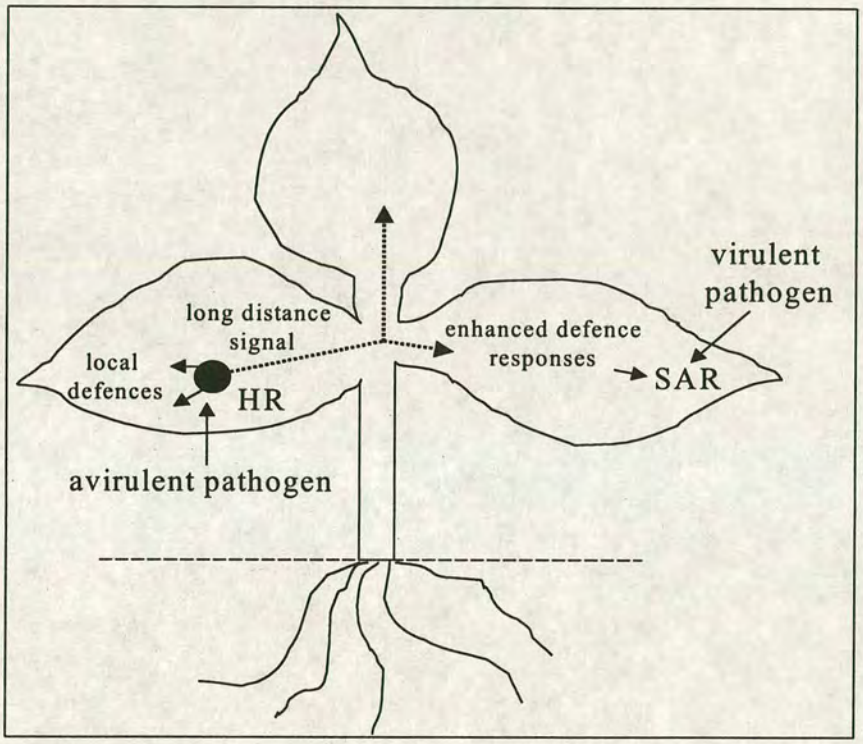

Fig.1.1. Schematic diagram of SAR induced by an avirulent pathogen.
Adapted from Ryals et al. (1994).

Signals and genes associated with acquired disease resistance

Classical SAR has been associated with salicylic acid (SA), but recently three other signalling molecules (jasmonic acid, ethylene and H_2O_2) have been intensively studied and all four molecules are thought to play very important roles in the induction of disease resistance (Dong 1998). Integration of signalling pathways leading to expression of defence genes and disease resistance are shown in Fig.1.2.

In Arabidopsis, SA-dependent SAR is associated with production of an HR by an avirulent pathogen, expression of *PR-1, PR-2* and *PR-5* genes (encoding pathogenesis-related proteins) and resistance to biotrophic pathogens such as *Pseudonmonas syringae* (Delaney et al. 1994), *Peronospora parasitica* (Lawton et al. 1995), *Erisiphe orontii* (Reuber et al. 1998) and turnip crinkle virus (TCV) (Kachroo et al. 2000, Uknes et al. 1993) (Fig.1.2). Resistance to necrotrophic fungal pathogens such as *Alternaria brassicicola, Botrytis cinerea, Fusarium oxysporum* and *Pythium* sp (Penninckx et al.1996, Thomma et al. 1998, Epple et al. 1995, Staswick et al. 1998) is associated with JA and ethylene signalling and expression of genes encoding antimicrobial peptides (Fig.1.2). In addition, it has been found that production of an HR by *B.cinerea* facilitates its infection of Arabidopsis (Govrin & Levine 2000). Thus, it is likely that the HR is only important for resistance to biotrophic pathogens (Fig.1.2). The roles of H_2O_2, SA, JA and ethylene in inducing expression of *PR* and antimicrobial genes will be discussed separately below.

The role of reactive oxygen species

One of the earliest responses of plant cells to pathogens is the production of reactive oxygen species (ROS). Known as the oxidative burst, O_2^- accumulates initially, which is rapidly dismutated to H_2O_2 either non-enzymatically or by the action of superoxide dismutase (Grant & Loake 2000, Lamb & Dixon 1997, Low & Merida 1996, Mehdy et al. 1996). An initial, weak burst of ROS is evoked within one hour in response to inoculation with either virulent or avirulent pathogens (Mehdy et al. 1996). However, with avirulent pathogens, the oxidative burst is biphasic, with a second massive burst of ROS produced after approximately three hours (Mehdy et al.1996). The second burst correlates with the formation of the HR and the establishment of disease resistance (Fig.1.2).

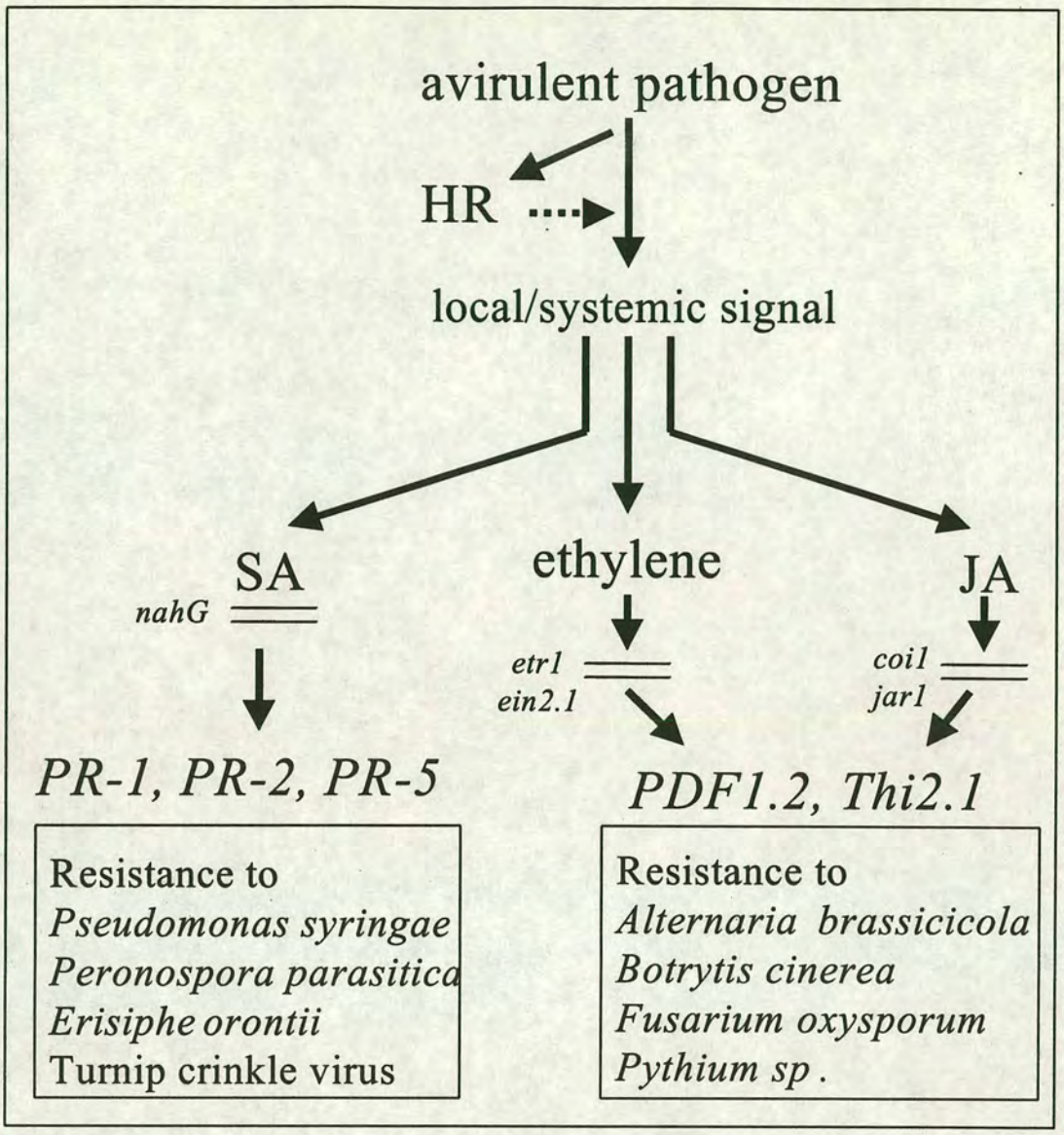

Fig.1.2. Model showing integration of signal transduction pathways leading to expression of defence-related genes and disease resistance in Arabidopsis.

Several sources are known to exist for the generation of ROS (reviewed in Grant & Loake 2000, Bolwell 1999). These include a plasmamembrane located NADPH oxidase, a cell wall peroxidase and amine, diamine and polyamine oxidase-type enzymes. Production of ROS is thought to involve a signal transduction cascade, including phosphorylation/dephosphorylation, G-proteins and Ca^{2+} influx (Grant & Loake 2000, Bolwell 1999).

ROS are thought to function in a number of different processes. One of these is a direct microbial effect, but there is little evidence suggesting that accumulated ROS are responsible for killing the invading pathogen (Bolwell 1999). However, it has been established that ROS are responsible for the oxidative cross-linking of cell wall hydroxyproline-rich proteins, thereby reinforcing the cell wall against pathogen secreted wall-degrading enzymes (Mehdy et al. 1996). It has also been proposed that ROS accumulation in response to avirulent pathogen inoculation initiates programmed cell death (PCD) in plant cells (Greenberg 1997) leading to the formation of the HR (Levine et al. 1994) (Fig.1.2). However, evidence is accumulating that PCD can be uncoupled from ROS production, and in a number of systems ROS is generated without cell death (Richberg et al. 1998). Nitric oxide (NO) has been shown to potentiate ROS-mediated induction of cell death in soybean cells, suggesting that NO also plays an important role in PCD and HR formation (Delledonne et al. 1998). A ROS-mediated systemic signalling network may also mediate the establishment of SAR (Alvarez et al.1998). Inoculation of Arabidopsis leaves with *P.syringae* pv *tomato (Pst)* expressing *avrRpt2* induced secondary oxidative bursts in distant tissues, leading to low-frequency systemic micro-HRs. Both the primary HR and the secondary micro-HRs were shown to be necessary for the development of SAR (Alvarez et al. 1998).

The role of salicylic acid

The plant metabolite salicylic acid (SA) plays an important role in SAR. SA increases in plants after pathogen infection in both local and systemic tissue, and studies have shown SA is required for the expression of a set of PR genes (Mauch-Mani & Metraux 1998, Delaney 1997, Sticher et al.1997, Ryals et al. 1994) (Fig.1.2). Moreover, application of SA or its analogues 2,6-dichloroisonicotinic acid (INA) (Kessmann et al. 1994) and benzothiadiazole (BTH) (Friedrich et al. 1996, Gorlach et al. 1996, Lawton et al. 1996) induces broad- spectrum disease resistance in plants.

SA is a product of phenylpropanoid metabolism, synthesised from phenylalanine which is initially converted to *trans*-cinnamic acid by phenylalanine ammonia lyase

(PAL) (Lee et al. 1995, Klessig & Malamy 1994). PAL is a key enzyme in the phenylpropanoid pathway that also yields phytoalexins, lignins and flavanoids (Klessig & Malamy 1994). Cinnamic acid is decarboxylated to benzoic acid which is converted to SA by 2-hydroxylation (Lee et al. 1995). A SA-binding protein has been purified and its cDNA cloned (Chen et al. 1993). This indicated that the protein is a catalase and that SA specifically inhibited this activity *in vitro*, leading to an increase in H_2O_2 (Chen et al. 1993). Thus, SA may act in SAR by elevating the levels of H_2O_2, which may induce the expression of *PR* genes (Chen et al. 1993). However, this hypothesis is unlikely as studies with transgenic tobacco expressing the H_2O_2-removing enzyme catalase in the antisense orientation (and thus unable to limit H_2O_2 accumulation) showed that SA was essential for accumulation of PR1 protein (Chamnongpol et al. 1998). Furthermore, this hypothesis is unlikely, as the oxidative burst and elevation of H_2O_2 levels occurs very early after pathogen inoculation and SA accumulates at a later time point (Lee et al. 1995).

Conclusive proof that SA is required for SAR comes from experiments with transgenic tobacco and Arabidopsis plants expressing the bacterial salicylate hydroxylase gene, *nahG* (Delaney et al. 1994). Salicylate hydroxylase converts SA to catechol, which is inactive in SAR signalling. Transgenic *nahG* plants accumulate a reduced amount of SA, do not express *PR-1* or display SAR in response to SAR-inducing organisms (Fig.1.2). In addition, *nahG* plants are also more susceptible to avirulent and virulent *Pseudomonas syringae* DC3000 pv *maculicola*, *Peronospora parasitica* (Delaney et al. 1994, Lawton et al. 1995), *Erisiphe orontii* (Reuber et al. 1998) and turnip crinkle virus (Kachroo et al. 2000) (Fig.1.2). Further proof for the role of SA in SAR comes from tobacco plants overproducing SA (Verberne et al. 2000). Tobacco plants were transformed with two bacterial genes coding for enzymes that convert chorismate into SA by a two-step process in chloroplasts. Transgenic plants showed a 500- to 1000-fold increase in SA accumulation, constitutive expression of acidic *PR* genes and enhanced resistance to a viral and a fungal pathogen (Verberne et al. 2000).

Evidence both for and against SA as the translocated signal in SAR have been reported (Sticher et al. 1997, Ryals et al. 1996, Klessig et al. 1994). *In vivo* SA-labelling studies have provided evidence that SA produced in tobacco mosaic virus (TMV)-infected tobacco leaves is transported to the upper, non-infected leaves of the plant, accounting for 70% of the accumulated SA (Shulaev et al. 1995). However, in cucumber infected with *P. syringae*, removal of the primarily infected leaf six hours after inoculation, which is before SA accumulates, still resulted in the expression of SAR (Rasmussen et al. 1991). In addition, TMV inoculation of *nahG* rootstocks resulted in very little SA accumulation in infected tissue, but SAR was established in the grafted wild type scion, indicating that translocation of the systemic signal was unaffected (Vernooij et al. 1994). Once SA accumulates, it is rapidly converted to β-0-D-glucosylsalicylic acid (SAG). SAG does not appear to be active in disease resistance and thus may either represent a detoxifying step in which SA levels are maintained below toxic concentrations, or an inert SA storage compound (Sticher et al. 1997, Ryals et al. 1996). Methyl salicylate, another metabolite of SA, is produced in large quantities in infected tissue (Seskar et al. 1998, Shulaev et al. 1997), and could function as an airborne signal in establishing SAR both in the same plant and in neighbouring plants (Shulaev et al. 1997).

In addition to the direct role played by SA in mediating disease resistance, evidence is also emerging for an indirect role, where SA potentiates the induction of local defence responses (Shirasu et al. 1997, Mur et al. 1996). When transgenic tobacco plants expressing the *GUS* reporter gene under the control of the asparagus PR10 promoter (*AoPR-1:GUS*) or *PAL3* promoter (*PAL3:GUS*) were hydroponically fed with 1 to 2 mM SA for 1 to 7 days, transgenic plants exhibited enhanced expression of both defence gene promoter fusions after wounding or infection with TMV or *P.syringae* pv *syringae* (Mur et al. 1996). In addition, it was found that inclusion of SA at physiological concentrations (50μM) in soybean cell suspensions potentiates an early step in the activation of local defence responses to an avirulent strain of *P.syringae* pv *glycinea* (Shirasu et al. 1997). An increase in H_2O_2 accumulation, *GST1* and *PAL1* expression, and hypersensitive cell death was visualised. This led to the conclusion that SA stimulates an agonist-dependent gain control operating at an

early step in the signal transduction pathway for induction of the hypersensitive response (Shirasu et al. 1997).

The role of jasmonic acid

Jasmonic acid (JA) and its volatile counterpart methyl jasmonate (Me-JA) (referred to collectively as jasmonates) are distributed throughout higher plants and effect many diverse processes (Creelman & Mullet 1997, Wasternack & Parthier 1997). These include petiole abscission, tendril coiling, fruit ripening, pollen germination, root growth and plant resistance to insects and pathogens (Creelman and Mullet 1997). JA is a 12-carbon fatty acid derivative, which is synthesised via the octadecanoid pathway from the 18-carbon substrate linoleic acid (Leon & Sanchez-Serrano 1999). The linoleic precursor is catalysed to linolenic acid by ω-3 fatty acid desaturases in the chloroplasts (Leon & Sanchez-Serrano 1999). Linolenic acid is converted in turn to 12-oxo-phytodienoic acid in a multi-step enzymatic process involving lipoxygenase, allene oxide synthase and allene oxide cyclase activity. JA synthesis proceeds in the cytoplasm with the action of 12-oxo-phytodienoic acid reductase and is followed by three rounds of β-oxidation which takes place in the peroxisomes (Leon & Sanchez-Serrano 1999). The majority of the genes corresponding to these enzymes are transcriptionally activated and some of them are also activated by JA, allowing for feed-back regulation of the biosynthetic pathway (Leon & Sanchez-Serrano 1999).

JA appears to play an important role as a 'master switch' in signal transduction pathways in response to insects and pathogens (Wasternack & Parthier 1997). Proteins encoded by JA-induced genes include antimicrobial peptides, phytoalexin biosynthetic enzymes, storage proteins and stress protectants (Wasternack & Parthier 1997). The JA-dependent wound-induced formation of proteinase inhibitors has been well characterised (Wasternack & Parthier 1997, Schaller & Ryan 1995). Proteinase inhibitors in tomato leaves (PIN1 and PIN2) accumulate systemically after herbivore attack and protect the plant against the digestive enzymes produced by the insect (Schaller & Ryan 1995). The systemic proteinase inhibitor- inducing factor has been proposed to be an 18 amino acid peptide termed systemin (Ryan

1992). Systemin is processed from a larger precursor protein termed prosystemin upon wounding, which is followed by the accumulation of linolenic acid and the subsequent production of jasmonic acid (Schaller & Ryan 1995). Both abscisic acid (Pena-Cortes et al. 1995) and ethylene (O'Donnell et al. 1996) are also required in this process. It has also been found that jasmonates are essential for insect defence in Arabidopsis (McConn et al. 1997). The Arabidopsis *fad3-2 fad7-2 fad8* triple mutant, which can not produce the ω-3 fatty acid desaturases required for conversion of linoleic acid to linolenic acid and are thus deficient in JA (McConn & Browse 1996), were infected with larvae of *Bradysia impatiens* (common fungal gnat). The mutant plants were extremely susceptible to attack by this insect species. However, prior application of Me-JA protected the plants, indicating that jasmonates play a vital role in mediating resistance against *Bradysia impatiens* (McConn et al. 1997).

Two jasmonic acid responsive Arabidopsis mutants have been identified. The first of these, *jar1* (*j*asmonic *a*cid *r*esistant 1) (Fig.1.2) showed decreased sensitivity to Me-JA inhibition of root elongation on agar medium containing 0.1μM Me-JA in comparison to wild-type seedlings (Staswick et al. 1992). Genetic data indicate that *jar1* defines a single recessive gene (Staswick et al. 1992). The second mutant, *coi1* (*co*ronatine-*i*nsensitive 1) (Fig.1.2), was selected on agar medium containing coronatine, a phytotoxin secreted by certain strains of *Pseudomonas syringae* and an analogue of JA (Feys et al. 1994). The *coi1* mutant is male sterile and also defines a single recessive gene (Feys et al. 1994). The *COI1* gene has been cloned, and found to encode a protein containing leucine-rich repeats and a degenerate F-box motif (Xie et al. 1998). These features are characteristic of proteins that ubiquinate proteins targeted for removal. Thus COI1 may function by degrading a repressor protein in the JA signal transduction pathway (Xie et al. 1998).

It has become apparent that jasmonates play an important role in the regulation of pathogen defences. It has been shown that jasmonates are part of the signal transduction pathway leading to the expression of the antimicrobial peptides *PDF1.2* and *Thi2.1* in Arabidopsis in response to the necrotrophic fungal pathogens *Alternaria brassicicola, Botrytis cinerea* and *Fusarium oxysporum* (Fig.1.2) (see

below). Jasmonate signalling is also important in resistance to the soil fungus *Pythium* sp (Fig.1.2) (Staswick et al. 1998, Vijayan et al. 1998). Both the *jar1* (Staswick et al. 1998) and *fad3-2 fad7-2 fad8* (Vijayan et al. 1998) mutants were extremely susceptible to root rot caused by *Pythium* sp, whereas wild type plants were not. JA has also been found to accumulate in tobacco after inoculation with *Pseudomonas syringae* pv. *phaseolicola* (Kenton et al. 1999). JA accumulates 3 to 9 hours after bacterial infection and is restricted to the developing HR lesion (Kenton et al. 1999). This contrasts with the systemic accumulation of JA observed in the Arabidopsis-*A.brassicicola* interaction (Penninckx et al. 1996).

The role of ethylene

The simple gas ethylene (C_2H_4) is a phytohormone affecting all stages of plant growth and development, including germination, senescence, abscission, flowering, fruit ripening in climacteric fruits and responses to various stresses (reviewed in Chang & Shockey 1999, Johnson & Ecker 1998, Solano & Ecker 1998, Kieber 1997). Ethylene is formed from methionine via S-adenosyl methionine and 1-aminocyclopropane-1-carboxylic acid (ACC) (Kende 1993). S-adenosyl methionine is converted to ACC by the enzyme ACC synthase, and ACC is converted to ethylene by ACC oxidase, which is also called the ethylene-forming enzyme (Kende 1993). Both enzymes are encoded by multigene families whose members are differentially regulated by external stimuli such as flooding, pathogen infection, wounding or internal stimuli such as germination, fruit ripening or senescence (Johnson & Ecker 1998). Some of these processes can also act through other hormones such as auxin and cytokinin (Johnson & Ecker 1998).

In addition to understanding the production of ethylene, considerable progress has also been made in the genetic and molecular dissection of the ethylene-response pathway. A number of loci involved in ethylene signalling have been identified on the basis of the isolation of ethylene-response mutants in Arabidopsis. These mutant screens were based on the 'triple response', a series of dramatic morphological changes undergone by seedlings when grown in the dark in the presence of ethylene. In Arabidopsis, the triple response includes the inhibition of hypocotyl and root

elongation, radial swelling of hypocotyl and root cells and exaggeration of the apical hook (Ecker 1995). Mutants that display a 'constitutive' triple response result either from ethylene overproduction (*eto1, eto2* and *eto3*) or constitutive activation of the ethylene response pathway (*ctr1*) (Guzman & Ecker 1990, Roman et al. 1995). Mutants unable to perceive or respond to ethylene include *etr1, etr2, ein2, ein3, ein4, ein5, ein6, ein7* and *eir1* (Bleeker et al. 1988, Roman et al. 1995). Genetic analysis of these mutants has indicated that they act in a linear pathway (Roman et al. 1995). In the current view, ethylene is perceived at the plasma membrane by a family of ethylene receptors that include ETR1, ETR2, EIN4, ERS1 and ERS2 (Stepanova & Ecker 2000, Chang & Shockey 1999, Solano & Ecker 1998, Kieber 1997). From the membrane, the signal is transduced to the nucleus through a series of proteins that include CTR1, EIN2, EIN5, EIN6 and EIN7 (Chang & Shockey 1999, Solano & Ecker 1998, Kieber 1997). The current model for ethylene signalling in Arabidopsis proposes that ethylene binding deactivates the receptors such that, in the absence of a positive regulatory signal from receptors, the CTR1 protein becomes inactive (Stepanova & Ecker 2000). The ethylene signal is further transduced through the positive regulator EIN2 (Stepanova & Ecker 2000). In the nucleus, the EIN3-family of transcription factors initiate the expression of the ethylene responsive genes (Stepanova & Ecker 2000, Chang & Shockey 1999, Solano & Ecker 1998).

Cloning of the *ETR1* gene uncovered its similarity to two-component histidine-kinase regulators that are sensors and transducers of environmental stimuli in bacteria (Chang et al. 1993). Two component receptors consist of a sensor protein with a histidine autokinase domain and a response regulator. Activation of the histidine-kinase promotes autophosphorylation of the histidine and a subsequent transfer of the phosphoryl group to an aspartate residue in the receiver domain of the response regulator protein (Chang et al. 1993). CTR1, which is a negative regulator of the ethylene response, acts downstream of the ethylene receptors and has similarity to the Raf family of mitogen-activated protein kinase kinase kinases (MAPKKKs) (Kieber et al. 1993). This suggests that the ethylene signal is propagated through a MAP kinase cascade. Cloning of *EIN2* indicates that it codes for a structurally novel protein with an amino-terminal integral membrane domain

that has similarity to the Nramp family of metal-ion transporters (Alonso et al. 1999). The *EIN3* gene codes for a nuclear-localised DNA binding protein (Chao et al. 1997).

Ethylene production often correlates with plant-pathogen interactions but its function in disease resistance and/or susceptibility is unclear and appears to be dependent on the pathogen species involved. The *etr1* and *ein2* mutants have been used in studies aimed at determining the role of ethylene accumulation in disease resistance. Bent and co-workers infected the Arabidopsis mutants *etr1* and *ein2* with avirulent *Pst* strains (Bent et al. 1992). Both mutants were resistant to the bacteria, suggesting that ethylene is not required for resistance against avirulent bacterial pathogens. In a further experiment where the mutants were infected with virulent *Pst*, *P.syringae* pv *maculicola* or *Xanthomonas campestris* pv *campestris, ein2* developed minimal disease symptoms whereas wildtype plants and *etr1* showed development of disease symptoms. However, virulent *Pst* grew to wildtype levels in *ein2*, indicating that the transduction of the ethylene signal as defined by this part of the pathway may be involved in pathogen-induced damage but not disease resistance. Further studies with the *etr1* and *ein2* mutants showed normal *PR* gene induction and *P.parasitica* resistance in response to SA, indicating that SA-induced SAR is independent of ethylene in Arabidopsis (Lawton et al. 1994). SAR induced in Arabidopsis by *Pst (avrRpt2)* against *P.parasitica* was not abolished in *etr1* or *ein2* plants, further indicating that ethylene is not required for SA dependent SAR (Lawton et al. 1995) (Fig.1.2). Ethylene also appears to play a role in the development of disease symptoms in tomato. The tomato mutant *Never ripe*, which is impaired in ethylene perception, and a transgenic tomato line expressing the ACC-deaminase gene, which directs degradation of ACC and thereby inhibits ethylene production, exhibited a reduction in disease symptoms in comparison to wild type after inoculation with virulent *Pst*, *X.campestris* pv *vesicatoria* or *Fusarium oxysporum* f sp *lycopersici* (Lund et al. 1998).

Further evidence that ethylene has different effects depending on the plant-pathogen interaction come from a number of different sources. Tobacco transformed with the

mutant *etr1* gene from Arabidopsis remained resistant to TMV, but lost resistance to the normally non-pathogenic soil-borne fungus *Pythium sylvaticum* (Knoester et al. 1998). Arabidopsis *ein2* mutant plants inoculated with *Botrytis cinerea* were more susceptible than wild-type plants (Fig.1.2), whereas no increased fungal growth was observed in *ein2* plants after inoculation with avirulent fungal pathogens *P.parasitica* or *Alternaria brassicicola* (Thomma et al. 1999a). In addition, *ein2.1* and *etr1* plants showed reduction of *PDF1.2* expression and enhanced susceptibility to the virulent bacterium *Erwinia carotovora* subsp. *carotovora* (Norman-Setterblad et al. 2000). However, these ethylene-insensitive mutants exhibited a normal SAR response to *P.parasitica* infection upon pre-treatment with harpin, the product of the *hrpN* gene of *Erwinia amylovora* (Dong et al. 2000). Studies with soybean mutants were also not clear-cut. Ethylene-insensitive soybean mutants were either more resistant or more susceptible to a range of bacterial and fungal pathogens (Hoffman et al. 1999).

Pathogenesis-related proteins

Associated with both local resistance to an avirulent pathogen and SAR are the expression of a number of genes which encode pathogenesis-related (PR) proteins (Ward et al. 1991). In tobacco, the set of PR proteins consists of at least nine families, comprising acidic forms of PR1 (PR1a, PR1b, PR1c), β-1,3-glucanase (PR2a, PR2b, PR2c), class II chitinases (PR3a and PR3b, also called PR-Q), hevein-like protein (PR4a and PR4b), thaumatin-like protein (PR5a and PR5b), acidic and basic isoforms of class III chitinase, an extracellular β-1,3-glucanase and the basic isoform of PR1 (Ryals et al. 1996, Ward et al. 1991). In general, accumulation of acidic PR proteins is dependent on SA (Ward et al. 1991). PR1, PR2, PR3, PR4 and PR5 show antimicrobial activity *in vitro*, with chitinases and β-1,3-glucanases showing antifungal activity (Sticher et al. 1997). The role of PR proteins in defence have been investigated using transgenic plants expressing the corresponding gene under the control of the cauliflower mosaic virus 35S promoter, and in some cases the transgenic plants were more resistant to pathogens (Sticher et al. 1997). In particular, over-expression of *PR-1* in tobacco increased resistance to *Peronospora tabacina* and *Phytophthora parasitica* var. *nicotianae* but not to *Ceronospora nicotianae* or *P. syringae* pv. *tabaci* (Alexander et al. 1993).

In Arabidopsis PR proteins dependent on the accumulation of SA comprise *PR-1, PR-2* and *PR-5*, with PR-1 being the predominant protein (Uknes et al. 1992) (Fig. 1.2). Acidic and basic chitinases (*PR3*) were isolated from Arabidopsis (Samac et al. 1990). Expression of the acidic chitinase gene was not observed in untreated plants or in plants treated with ethylene or SA, whereas the basic chitinase gene was expressed constitutively in roots and systemically upon application of ethylene (Samac et al. 1990). A hevein-like gene, with 70% homology to tobacco *PR4*, was isolated and was found to be inducible by SA, ethylene and infection by turnip crinkle virus (Potter et al. 1993). In addition, expression of both the basic *PR3* and *PR4* in Arabidopsis was induced by application of methyl jasmonate or inoculation with *A.brassicicola* (Thomma et al. 1998).

The role of antimicrobial peptides

The production of antimicrobial peptides is thought to be an ancient and widespread defence strategy. Thionins and plant defensins are examples of antimicrobial peptides that have been isolated from plants. Thionin genes, originally identified in barley, have also been identified in Arabidopsis and are toxic to several phytopathogenic bacteria and fungi (Epple et al. 1995). Plant defensins also have antifungal activity (Broekaert et al. 1995). Recently, a re-classification of PR-proteins has included plant defensins and thionins, and has listed them as PR-12 and PR-13 respectively (Van Loon & Van Strien 1999).

A search of Arabidopsis expressed sequenced tags (ESTs) identified two putative defensins, called plant defensin 1.1 (PDF1.1) and PDF1.2 (Penninckx et al. 1996). *PDF1.1* was expressed in silliques and seeds, while *PDF1.2* was expressed in response to infection by the compatible fungus *Alternaria brassicicola* in both locally infected and systemic leaves (Penninckx et al. 1996). Expression of *PDF1.2* correlated with an increase in expression of jasmonic acid in both types of leaves (Penninckx et al. 1996). *PDF 1.2* was not expressed in response to SA or INA treatment, but was expressed in response to Me-JA or ethylene (Fig.1.2).

Two cDNAS encoding thionin preproteins have been isolated from Arabidopsis, and have been designated *Thi2.1* and *Thi2.2* respectively (Epple et al. 1995). *Thi2.2* is expressed at low levels in seedlings and rosette leaves, whereas *Thi2.1* is expressed in leaves, and very highly in flowers and silliques. Application of methyl jasmonate, silver nitrate and infection with *Fusarium oxysporum f.* sp. *matthiolae* induced *Thi2.1* to high levels (Fig.1.2), but had no effect on *Thi2.2* expression (Epple et al. 1995). Application of SA to Arabidopsis plants did not induce *Thi2.1* expression, indicating that this gene is also induced via a signal transduction pathway different to that for PR proteins (Epple et al. 1995). Over-expression of the *Thi2.1* gene in the susceptible Arabidopsis ecotype Columbia (Col-0) resulted in enhanced resistance to *F. oxysporum f.* sp. *matthiolae* (Epple et al. 1997a). In addition, it was found that *Thi2.1* expression correlates to *F. oxysporum* resistance: the resistant ecotypes Mt-0 and UK-4 had 5 to 10 times higher accumulation of *Thi2.1* in comparison to the susceptible ecotypes Col-0, Landsberg erecta (L*er*) and Wassilewskija (Ws) (Epple et al. 1998).

Rhizobacteria-mediated Induced Systemic Resistance

Some non-pathogenic rhizobacteria can induce systemic resistance in plants that is phenotypically similar to pathogen-induced SAR. Rhizobacteria-mediated induced systemic resistance (ISR) has been demonstrated against fungi, bacteria and viruses in Arabidopsis, bean, carnation, cucumber, radish, tobacco and tomato under conditions in which the inducing bacteria and the challenging pathogen remain spatially separated (Pieterse & Van Loon 1999, Van Loon et al. 1998). Bacterial strains differ in their mode of action and the extent of their ability in inducing ISR, and plants differ in their ability to express ISR. Bacterial determinates of ISR include lipopolysaccharides, sideophores and SA (reviewed in van Loon 1998). Most of the ISR-inducing rhizobacteria belong to the fluorescent *Pseudomonas* sp. A model system has been developed in which *Pseudomonas fluorescens* WCS417r is used to induce ISR in Arabidopsis against *Pst* and *F. oxysporum* fsp *raphani* (Pieterse et al. 1996). ISR was found to be effective in *nahG* plants and did not induce expression of the *PR-1, PR-2* and *PR-5* genes, indicating that SA is not needed for ISR. Therefore, ISR may be induced via a different pathway to that of

SAR (Fig.1.3, see below) (Van Wees et al. 1997, Pieterse et al. 1996). Studies of ISR against *Pst* using *jar1*, the ethylene insensitive mutants *etr1*, *ein2* through *ein7* and *eir1*, and the SAR regulatory mutant *npr1* (see below), indicated that components of the jasmonate and ethylene response network are engaged successively in inducing resistance (Fig.1.3, see below) (Knoester et al. 1999, Pieterse et al. 1998). Furthermore, ethylene responsiveness is required at the site of application of the inducing rhizobacteria and the complete known signal transduction pathway of ethylene is required for ISR (Knoester et al. 1999). ISR against *Pst* was abolished in the *npr1* mutant, indicating that NPR1 regulates both SAR and ISR (Pieterse et al. 1998) (Fig.1.3, see below). It has also been shown that the simultaneous activation of ISR and SAR results in an additive effect on the level of resistance against *Pst* (Van Wees et al. 2000). No ISR marker proteins or substantial changes in gene expression have been identified to date, but ISR was found to stimulate the expression of the jasmonate-inducible vegetative storage gene *Atvsp* upon challenge with *Pst* (Van Wees et al. 1999). A single dominant gene, *ISR1*, has been identified which is required for both ISR and basal resistance against *Pst*, suggesting that a high level of basal resistance is required for induction of ISR against *Pst* in Arabidopsis (Ton et al. 1999).

1.5. SAR signal transduction network

The production of mutants with abnormalities in disease resistance makes it possible to define the signal transduction network underlying SAR. Evidence is emerging for both a SA-dependent and a SA-independent, jasmonate- and ethylene-dependent signal transduction pathway leading to the establishment of disease resistance to different pathogens (Fig.1.2) (Thomma et al. 1998, Bowling et al. 1997, Ryals et al. 1996, Penninckx et al. 1996). Fig.1.3 outlines the current model of the defence response signalling network and the positioning of genes identified in mutant screens.

Mutants in the transduction of R-gene-mediated signals

The *ndr1* (*n*onrace-specific *d*isease *r*esistance) mutant, produced in the Arabidopsis ecotype Col-0, is susceptible to *Pst*DC3000 carrying any one of four *avr* genes: *avrRpm1, avrRpt2, avrB* and *avrPph3*, as well as the Emyo2 and Emwa1 isolates of *P. parasitica* (Century et al. 1995). Col-0 plants are resistant to the Emyo2 and Emwa1 isolates, and require the presence of the *RPP4* (resistance to *P. parasitica 4*) locus for resistance (Holub et al. 1994). This indicates that the *NDR1* gene product is a part of a common signal transduction pathway that mediates resistance to both bacterial and oomycete pathogens, and is required for the function of the *RPM1, RPS2, RPS5* and *RPP4* genes. The *NDR1* gene product has been placed between the HR and SA in the signal transduction pathway (Fig.1.3). The *NDR1* gene has been isolated and cloned, and the predicted amino acid sequence suggests that NDR1 may be associated with the plasma membrane (Century et al. 1997).

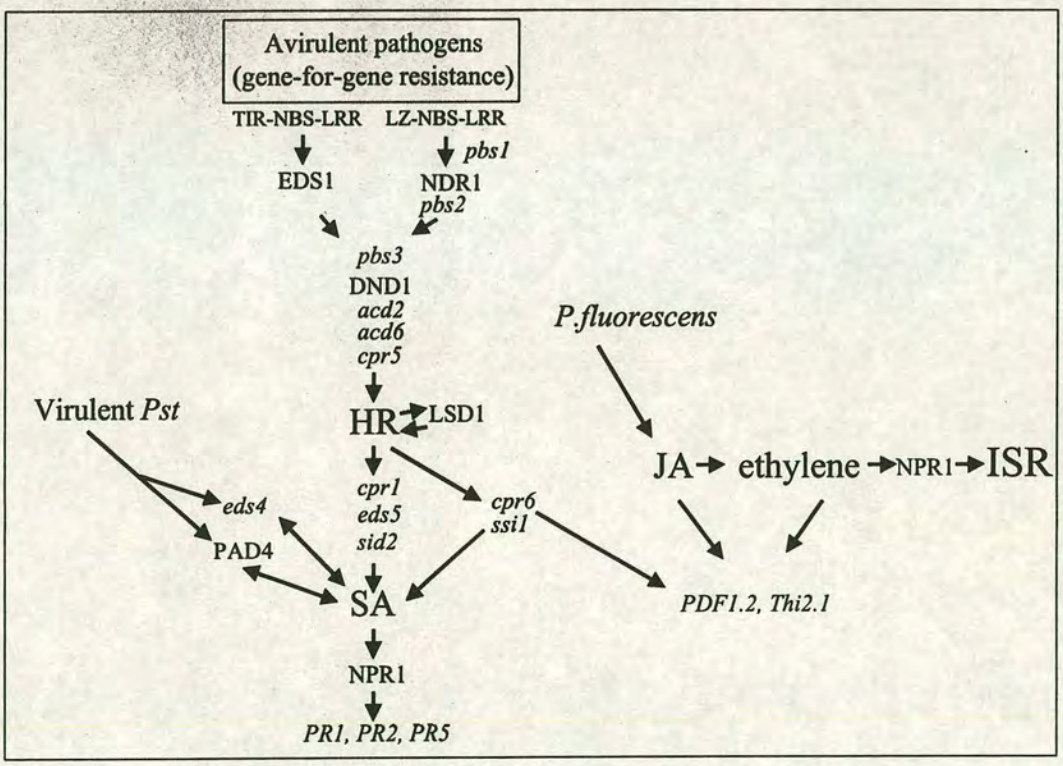

Fig.1.3. Current model of the SAR signal transduction network in Arabidopsis.

Adapted from Glazebrook (1999) and Feys & Parker (2000). The position of cloned genes and the site of action of their predicted proteins are shown in capitals. Mutants, representing currently uncloned genes, are shown in lower case. For simplicity, not all mutants mentioned in the text are represented in this model.

The *eds1* (*e*nhanced *d*isease *s*usceptibility *1*) mutant, produced in the Arabidopsis ecotype Ws, supported heavy sporulation of the *P.parasitica* isolates Emoy2, Cala2, Wela3 and Noco2 normally showing an incompatible interaction with Ws (Parker et al. 1996). In Ws, the *RPP1, RPP10, RPP12* and *RPP14* loci are required for resistance to the Emoy2, Cala2, Wela3 and Noco2 isolates respectively (Holub et al. 1994). Further alleles of *eds1* were isolated in the Arabidopsis ecotype L*er* (Aarts et al. 1998). Studies with the *eds1.2* and *eds1.3* mutants indicated that *RPP5*-mediated resistance to *P.parasitica* Noco2 in Landberg erecta was lost, as was *RPS4*-mediated resistance to *P.syringae* pv *tomato* (*avrRps4*) (Aarts et al. 1998). Aarts and co-workers also showed that *RPP4*-mediated resistance to *P.parasitica* Emwa1 in a Col-0 *eds1* mutant was lost (Aarts et al. 1998). This indicates that the recessive *EDS1* locus is required for the function of the *RPP1, RPP4, RPP5, RPP10, RPP12, RPP14* and *RPS4* genes. The Ws-*eds1* mutant did not show compromised *RPM1*-mediated resistance to the corresponding bacterial avirulence gene *avrB* (Parker et al. 1996), but demonstrated resistance to both compatible and incompatible *P.parasitica* strains after treatment with INA (Parker et al. 1996) and showed *PR-1* expression after treatment with SA but not *Pst* (*avrRps4*) (Falk et al. 1999). This indicates that *EDS1* is a necessary component of resistance mediated by a number of *R* genes, and that EDS1 functions upstream of SA accumulation in the SA-dependent SAR signal transduction pathway (Fig.1.3). The *EDS1* gene has been cloned and found to encode a novel protein (Falk et al. 1999). However, it has similarity in its amino-terminal portion to the catalytic site of eukaryotic lipases belonging to the L-family (Falk et al. 1999), suggesting that EDS1 functions by hydrolyzing a lipid molecule. It is thus possible that EDS1 may be involved in processing JA-related fatty acid intermediates, or it may define an additonal lipid-based signal transduction pathway (Falk et al.1999).

It has been shown that the *EDS1* and *NDR1* gene products are required for the function of different resistance genes (Aarts et al. 1998). *EDS1* is required for *RPP1, RPP5, RPP10, RPP12, RPP14* (which confer resistance to *P.parasitica*), and *RPS4* which confers resistance to *Pst*(*avrRps4*) whereas *NDR1* is not required for the

function of these *R* genes (Aarts et al. 1998). Conversely, three *NDR1*-dependent *R* loci, *RPS2, RPM1* and *RPS5*, operate independently of *EDS1* (Aarts et al. 1998). Presented results for *RPP4* are unclear and do not correlate (Aarts et al. 1998, Century et al. 1995). Interestingly, the *RPP1, RPP5* and *RPS4 EDS1*-dependent *R* genes encode proteins of the TIR-NBS-LRR class, whereas *NDR1*-requiring *R* genes *RPS2, RPM1* and *RPS5* belongs to the LZ-NBS-LRR class (Table1.1, Aarts et al. 1998). This indicates that at least two *R*-gene mediated pathways are present in Arabidopsis and implies that the requirement for either EDS1 or NDR1 is governed by R protein structure (Aarts et al. 1998) (Fig.1.3). Further studies of the *RPP7* and *RPP8* loci (which also confer resistance to *P.parasitica*) have shown that neither *EDS1* nor *NDR1* are required for resistance mediated by these loci (McDowell et al. 2000). However, *RPP7* and *RPP8*-mediated resistance is weakly suppressed in the *eds1 ndr1* double mutant, suggesting that they operate additively through EDS1, NDR1 and other as yet unidentified signalling components (McDowell et al. 2000).

A further three putative signal transduction genes involved in *R* gene-specific disease resistance in Arabidopsis have been reported (Warren et al. 1999). Mutant plants were screened for loss of *RPS5*-specified resistance and five *pbs* (*avrPphB* susceptible) mutants, compromising three complementation groups, were uncovered. Mutations in *PBS1* blocked *RPS5* resistance only, and had no effect on resistance specified by other *R* genes. This indicates that PBS1 may be closely associated with the RPS5 / AvrPphB interaction (Warren et al. 1999). The *pbs2* mutant appears to suppress the same set of *R* genes as *ndr1* (*RPS2, RPS5* and *RPM1*) (Warren et al.1999) (Fig.1.3). The *ndr1* and *pbs2* mutations are not allelic and it is likely that the products of these two genes are closely associated in the same signal transduction pathway (Fig.1.3). The *pbs3* mutation partially suppressed four *P. syringae R* genes (*RPS2, RPS5, RPM1* and *RPS4*), the *RPP* genes and allowed higher growth of virulent *Pst* (Warren et al. 1999). This indicates that the *PBS3* gene product functions in a pathway involved in restricting both avirulent and virulent pathogens, and may operate downstream of both EDS1 and NDR1 (Warren et al. 1999) (Fig.1.3).

Isolation and characterisation of the *dnd1* (*d*efence, *n*o *d*eath *1*) mutant has provided evidence that the HR can be separated from gene-for-gene resistance in Arabidopsis (Yu et al. 1998). Mutant *dnd1* plants infected with avirulent *Pst* carrying either the *avrRpt2, avrRpm1* or *avrB* genes did not form a HR, but resistance to the avirulent bacteria was seen. Additionally, SA accumulated to higher levels and *PR-1* and *PR-2* were expressed constitutively (Yu et al. 1998). Mutant *dnd1* plants also exhibited enhanced resistance to virulent *Pst*, *Xanthomonas campestris*, *P.parasitica, Erisiphe orontii* and tobacco ringspot virus. It is likely that the DND1 product functions upstream of both SA accumulation and the HR in the defence response signalling network (Fig.1.3). Recently *DND1* has been cloned and it's predicted protein structure encodes a cyclic nucleotide-gated ion channel (Clough et al. 2000). Further mutants exhibiting an altered HR have been isolated including *ihr1* (*i*ntermediate *hr1*) (Yu et al. 2000). The *ihr1* mutant also displays elevated resistance to a wide range of pathogens, accumulates SA to higher levels and expresses *PR-1* and *PR-2* to higher levels (Yu et al. 2000).

Mutants expressing constitutive SAR

Different and independent screens have been used to identify mutants expressing SAR constitutively (Lawton et al. 1993, Bowling et al. 1994, Silva et al. 1999). In the first of these, RNA was isolated from EMS-mutagenised M2 seedlings using northern blots and hybridized to SAR gene probes (Lawton et al. 1993). This led to the discovery of *c*onstitutively *i*mmune *m*utants (*cim*), which show constitutive expression of *PR-1, PR-2* and *PR-5,* and resistance to pathogens. The second method entailed screening mutant transgenic plants containing the *BGL2(PR-2)* promoter fused to the marker gene β-glucoronidase (GUS) for constitutive GUS expression (Bowling et al. 1994). To date, the isolation and characterisation of three non-allelic *cpr* (*c*onstitutive expresser of *PR* genes) mutants have been reported: *cpr1* (Bowling et al. 1994), *cpr5* (Bowling et al. 1997) and *cpr6-1* (Clarke et al. 1998).

In addition to constitutive GUS expression, *cpr1* shows elevated expression in northern blot analysis of the *PR* genes, is resistant to *P. parasitica* Noco2 and *Psm*ES4326 and is associated with high endogenous levels of SA (Bowling et al.

1994). Progeny of a *cpr1nahG* double mutant do not produce SA and do not show the *cpr1* phenotype, indicating that the *CPR1* gene product acts upstream of SA in the SAR signal transduction pathway (Bowling et al. 1994) (Fig.1.3). The *cpr5* mutant displays spontaneous HR lesion formation and reduced trichome development in addition to the same characteristics as *cpr1*, which indicates that the *CPR5* gene product acts upstream of both HR formation and SA in the SAR pathway (Fig.1.3, Bowling et al. 1997). However, the *cpr5npr1* double mutants (*npr1* is incapable of establishing SA-dependent SAR, see below) continue to display resistance to *P. parasitica* Noco2 (Bowling et al. 1997). In addition, *cpr5* plants display elevated expression of *PDF1.2* (Bowling et al. 1997). This led to the conclusion that the *cpr5* mutation regulates constitutive expression of both a SA/NPR1 dependent pathway and a SA/NPR1 independent pathway, and that the *CPR5* gene is operating upstream of the HR and the branchpoint between the two pathways (Bowling et al.1997) (Fig.1.3). In a screen for suppressor mutations that restored resistance to a susceptible line carrying a mutation in the *RPS2* resistance gene, the *cpr5-2* mutant was isolated (Boch et al. 1998). Resistance gene-mediated defences, including the HR, restriction of *Pst* growth and induction of *PR-1* gene expression were observed in *cpr5-2* plants. Additionally, *RPS2- avrRpt2* mediated induction of *PR-1* expression was enhanced (Boch et al. 1998). Unlike *cpr1* and *cpr5* which are recessive mutants (Bowling et al. 1994, Bowling et al. 1997), *cpr6-1* is a dominant mutant (Clarke et al. 1998). The mutant *cpr6-1* displays constitutive expression of the *PR* and *PDF1.2* genes and resistance to *Psm*ES4326 and *P. parasitica* Noco2 (Clarke et al. 1998). Although bacterial resistance is not observed, *PR* gene expression is detected in the *cpr6-1npr1* double mutant, which is suppressed when SA is removed by crossing with a *nahG* plant (Clarke et al. 1998). This indicates that *PR* gene expression has been uncoupled from the *NPR1* gene product and bacterial resistance in the *cpr6-1* mutant. Hence, *CPR6* may regulate multiple signal transduction pathways (Fig.1.3) (Clarke et al.1998).

The *cep* T-DNA mutant (*c*onstitutive *e*xpression of the *P*R1 gene) was isolated in the Ws ecotype background (Silva et al. 1999). The *cep* mutant displays constitutive expression of the *PR* genes, elevated levels of SA accumulation, spontaneous

development of HR-like lesions and enhanced resistance to virulent *Psm* and *P.parasitica* Emwa (Silva et al. 1999). Although the *cep* phenotype segregated as a single recessive trait in the Ws genetic background, analysis of segregating progeny from crosses to other ecotypes revealed that the *cep* phenotype was due to mutations in two genes, designated *cpr20* and *cpr21* (Silva et al. 1999).

Lesion mimic mutants

Many mutants have been uncovered that spontaneously form HR lesions and some of these also expresses the *PR* genes, accumulate SA and express pathogen resistance (reviewed in Delaney 1997, Glazebrook et al.1997a). Simultaneous HR formation and SAR expression may come about because the plant is capable of falsely perceiving the pathogen. Included in this class are the *lsd* mutants (*l*esion *s*timulating *d*isease response): *lsd1, lsd2, lsd3, lsd4, lsd5* (Dietrich et al.1994), *lsd6* and *lsd7* (Weymann et al. 1995) and *acd* (*a*ccelerated *c*ell *d*eath) mutants (Greenberg et al. 1994, Rate et al 1999, Greenberg et al. 2000). The *acd2* recessive mutant forms lesions on older leaves only, so plants can be studied both before and after lesion development (Greenberg et al. 1994). These studies have shown that lesion formation is necessary for SA accumulation, *PR* gene expression and bacterial resistance (Greenberg et al. 1994). The *ACD2* gene product has been placed upstream of the HR (Fig.1.3). The *acd6* mutant has been characterised more recently (Rate et al. 1999). This dominant gain-of-function mutant is dwarfed in size, displays constitutive *PR-1* gene expression and shows increased resistance to both virulent and avirulent *Pst* (Rate et al. 1999). These phenotypes are suppressed in *acd6 nahG* plants, but are hyperactivated in *acd6 nahG* plants treated with BTH, suggesting that SA may be acting with a second defence signal (Rate et al. 1999). The *acd6* phenotypes are also suppressed in *acd6 npr1* plants, indicating that *acd6* acts through *npr1* (Rate et al. 1999). The ACD6 product is thus thought to be necessary for the activation of the SA/NPR1-dependent part of the signalling network, and is also required for the activation of the unknown second signal (Fig.1.3). Characterisation of the recessive *acd5* mutant further indicated that spontaneous lesion formation, SA accumulation and *PR-1* expression are correlated (Greenberg et al. 2000). However, *acd5* plants were not more resistant to *P.syringae*

than wild-type plants, indicating that in *acd5*, cell death and defence-related processes have been uncoupled from disease resistance (Greenberg et al. 2000).

The *lsd1* mutant differs from the others in that once HR lesions have formed, they spread and destroy the leaf by 'runaway' cell death, whereas in the other mutants the spread of the lesion is limited (Dietrich et al. 1994). It was found that superoxide accumulation is necessary for lesion spread in *lsd1* mutants (Jabs et al. 1996). Thus, the runaway cell death phenotype seen in *lsd1* plants appears to reflect an abnormal response to superoxide accumulation (Jabs et al. 1996). The *LSD1* gene has been cloned and the predicted LSD1 protein encodes a zinc finger protein, which suggests a role in transcriptional activation (Dietrich et al. 1997). Consequently, it was proposed that LSD1 functions in the regulation of transcription by either repressing a death pathway or activating a death inhibiting pathway (Dietrich et al. 1997) (Fig.1.3). More recently, it was shown that LSD1 regulates SA induction of a CuZn superoxide dismutase (SOD) (Kliebenstein et al. 1999). Thus, the spreading lesion phenotype in the *lsd1* mutant may be due to the lack of upregulation of a CuZn SOD responsible for detoxifying accumulating superoxide before the superoxide triggers cell death (Kliebenstein et al. 1999).

The *lsd2, lsd4, lsd6* and *lsd7* mutants were crossed with *nahG* plants in order to observe what happens in these mutants when SA accumulation is removed (Weymann et al. 1995, Hunt et al. 1997). Although *PR* gene expression and pathogen resistance were suppressed in the progeny of these crosses, the mutants differed in the formation of the HR lesion phenotype: *lsd2nahG* and *lsd4nahG* continued to form lesions (Hunt et al. 1997) whereas *lsd6nahG* and *lsd7nahG* did not (Weymann et al. 1995). In addition, application of SA or INA to the *lsd6nahG* plants initiated HR lesion formation (Weymann et al. 1995). This result could be explained by a feedback loop in SAR signalling such that HR lesions cause SA accumulation but other factors downstream of SA may also potentiate lesion formation (Weymann et al. 1995).

The *lsd5* mutant was used to isolated new mutations that suppress its cell death phenotype. Nine cell death suppressers were identified and were designated *phx* for the mythological bird *Ph*oenix that arose from its ashes (Morel & Dangl 1999). Four strong suppressers of cell death and constitutive *PR-1* were isolated (*phx2, phx3, phx6* and *phx11.1*). All four *phx* mutants showed enhanced susceptibility to avirulent *P.parasitica*, but only *phx2* and *phx3* were more susceptible to avirulent *Pst* and virulent *P.parasitica* (Morel & Dangl 1999). It is thus likely that the PHX2 and PHX3 products define common regulators of cell death and disease resistance.

Mutants incapable of expressing SA-dependent SAR

Three independent screens were used to identify mutants incapable of expressing SA-dependent SAR (Cao et al. 1994, Delaney et al. 1995, Shah et al. 1997). In the first of these, the *npr1* (*n*on-expresser of *PR* genes) was isolated (Cao et al. 1994). Mutant transgenic plants containing the *BGL-2*-GUS cassette were sprayed with SA or INA and screened for GUS activity (Cao et al. 1994). *npr1* mutants failed to express GUS and *PR-1* (Cao et al.1994). In the second screen, the *nim1* (*n*on-inducible *im*munity) mutant was isolated, which failed to develop resistance to *P. parasitica* in response to INA pre-treatment (Delaney et al. 1995). The *sai1* (*s*alicylic *a*cid-*i*nsensitive) mutant was isolated in the third screen (Shah et al. 1997). This screen utilised the SA-inducible expression of the Arabidopsis *tms2* gene under the control of the *PR-1a* promoter, which confers sensitivity to α-naphthalene acetamide (α-NAM) and results in inhibition of root growth (Shah et al. 1997). The *sai1* mutant is insensitive to α-NAM and does not express the *PR-1, PR-2* and *PR-5* genes in response to SA. The *npr1, nim1* and *sai1* mutants were shown to be alleles of the same recessive gene in complementation studies (Delaney et al. 1997, Shah et al. 1997). Plants with *npr1/nim1/sai1* mutations still accumulate SA in response to pathogen infection (Cao et al. 1994, Delaney et al. 1995). This indicates that the *NPR-1/NIM-1/SAI1* gene (designated *NPR1*) acts in the SAR signal transduction pathway downstream of SA (Fig.1.3).

NPR-1/NIM-1 was cloned by two separate map-based cloning projects (Cao et al. 1997, Ryals et al. 1997). From DNA sequence analysis, it appears that *NPR-1/NIM-*

1 encodes a novel protein containing ankyrin repeats, which implies a role in protein-protein interactions (Cao et al. 1997). Ryals and co-workers (Ryals et al. 1997) also detected homology to the mammalian transcription factor inhibitor IκB, which has been implicated in the immune response. The NPR1 protein has been expressed in Arabidopsis under the control of the CaMV35S promoter (Cao et al. 1998). The resulting transgenic plants expressed SAR genes more strongly upon induction and showed dramatic resistance to *P.syringae* and *P.parasitica* (Cao et al. 1998).

Two mutant screens have been conducted in order to uncover suppressers of the *npr1* mutation. The dominant *ssi1* (*s*uppresser of *S*A *i*nsensitivity *1*) mutant was isolated in the first of these screens as a suppresser of *npr1-5* (previously *sai1*) (Shah et al. 1999). In *ssi1* plants, which are small and spontaneously develop HR-like lesions, *PR-1, PR-2, PR-5* and *PDF1.2* are expressed constitutively, SA accumulates to elevated levels and plants remain resistant to *Pst* infection (Shah et al. 1999). These phenotypes remain in *ssi1 npr1* plants, but are abolished in *ssi1 npr1 nahG* plants, indicating that they are dependent on SA (Shah et al. 1999). In the second screen, the recessive *sni1* (*s*uppresser of *n*pr1 *i*nducible *1*) mutant was isolated as a suppresser of *npr1-1* (Li et al. 1999). The *sni1* mutant, which is smaller than wild type plants, expressed *PR-1, PR-2* and *PR-5* to wild type levels, accumulated SA and displayed resistance to *Psm* and *P.parasitica* only after application of SA (Li et al. 1999). The expression of *PDF1.2* was not recorded. The *SNI1* gene was cloned by map-based cloning and was found to contain a novel leucine-rich nuclear protein (Li et al. 1999).

Using NPR1 as a bait in a yeast two-hybrid screen, a subclass of transcription factors in the basic leucine zipper protein (bZIP) family were isolated (Zhou et al. 2000, Zhang et al. 1999). Small differences were found in the extent of interaction between different members of the bZIP family and NPR1 (Zhou et al. 2000, Zhang et al. 1999). It was shown that these bZIP transcription factors interacted specifically with NPR1 in yeast and *in vitro,* and that point mutations that abolished NPR1 function in Arabidopsis also abolished interactions between NPR1 and the transcription factors in the yeast two-hybrid assay (Zhang et al. 1999). It was also

shown in a gel mobility shift assay that the purified bZIP transcription factor protein, AHBP-1b, bound specifically to a SA-responsive promoter element in the Arabidopsis *PR-1* gene. This indicates that NPR1 may regulate *PR-1* expression by interaction with bZIP transcription factors (Zhou et al. 2000, Zhang et al. 1999). A model has been proposed whereby SNI1 represses the expression of *PR* genes in the absence of SA (Li et al. 1999). When SA is introduced, it activates NPR1, which represses the SNI1 represser, allowing transcription of *PR* genes. SA also activates a second factor, possibly the bZIP transcription factors, that can also allow transcription of *PR* genes (Li et al. 1999).

SA-independent SAR

To date, no Arabidopsis mutants have been specifically isolated as being important in jasmonate- and /or ethylene-dependent SAR signalling. However, by studying the JA and ethylene-dependent expression of *PDF1.2* and *Thi2.1* in combination with other SAR signalling mutants, it has become apparent that SA-independent signal transduction plays a role in establishing resistance against necrotrophic fungal pathogens (Thomma et al. 1998, Penninckx et al. 1996, Epple et al. 1995).

PDF 1.2 expression was not affected by the *nahG* transgene, or by the *npr1* or *cpr1* mutations (Penninckx et al. 1996). However, the *ein2* or *coi1* mutations greatly reduced *PDF 1.2* expression both locally and systemically (Penninckx et al. 1996), indicating that both jasmonates and ethylene, and not salicylic acid, are important in the signal transduction pathway leading to *PDF1.2* expression. More recently, in further studies where the *coi1* and *ein2* mutants were treated with either ethylene or Me-JA, it was established that both the ethylene and jasmonate signalling pathways need to be triggered concominantly, not sequentially, in order to activate *PDF1.2* expression upon *A. brassicicola* infection (Penninckx et al. 1998). *PDF1.2* was also expressed to a high level in Arabidopsis seedlings infected with the compatible necrotrphic fungus, *Fusarium oxysporum f.* sp. *matthiolae* (Epple et al. 1997b). In this study, *PDF1.2* expression was induced in seedlings treated with Me-JA and silver nitrate, but not by SA or the ethylene-producing compound, ethephon. Furthermore, it was found that *PDF1.2* was expressed in naïve mature rosette leaves,

but not in naïve seedlings (Epple et al. 1997b). It is possible that *PDF1.2* is expressed at a very low level in rosette leaves, and so was only detected by Epple and co-workers (1997b) but not by Penninckx and co-workers (1996). However, it is quite clear in both studies that *PDF1.2* expression is induced to higher levels by a range of compatible necrotrophic fungi.

PDF1.2 expression was further investigated by activity studies of the *PDF1.2* promoter (Manners et al. 1998, Mitter et al. 1998). The *PDF1.2* promoter was linked to the β-glucoronidase (GUS) reporter gene as a translational fusion and transformed into Arabidopsis (Manners et al. 1998) or tobacco (Mitter et al. 1998). Challenge of the transgenic Arabidopsis plants with *A.brassicicola* or the necrotrophic fungal pathogen *Botrytis cinerea* resulted in both local and systemic GUS expression (Manners et al. 1998). Wounding had no effect on GUS expression, while treatment with either jasmonic acid or the reactive oxygen producing compound paraquat strongly induced GUS activity (Manners et al. 1998). SA or ethylene application did not result in GUS expression (Manners et al. 1998). In contrast to the transgenic Arabidopsis, the *PDF1.2:GUS* transgenic tobacco expressed GUS in response to jasmonic acid and ethylene (Mitter et al. 1998), indicating that the ability of ethylene to induce *PDF1.2* expression is genotype-dependent. GUS expression was also strongly induced by inoculation of the transgenic tobacco plants with *Phytophthora parasitica*, *Cercospora nicotianae* and TMV (Mitter et al. 1998).

In order to study the expression of the *Thi2.1* gene, the cognate promoter was fused to the GUS gene and transformed into Arabidopsis (Vignutelli et al. 1998). Systemic and local GUS expression could be induced by *F. oxysporum* f.sp. *matthiolae* or by wounding in plants and in young seedlings by Me-JA application (Vignutelli et al. 1998), silver nitrate and sorbitol (Bohlman et al. 1998). Treatment of transgenic plants with inhibitors of JA biosynthesis led to reduced *Thi2.1* expression, indicating that JA is an important step leading to *Thi2.1* expression. The *coi1* and *fad3-2 fad7-2 fad8* mutants were crossed separately to the *Thi2.1:GUS* transgenic line and GUS activity was analysed in the progeny plants (Bohlman et al. 1998). After methyl jasmonate application, no GUS activity was seen in the *coi1*

background, whereas GUS activity was seen in the *fad3-2 fad7-2 fad8* mutant, confirming that the JA-dependent octadecanoid pathway regulates *Thi2.1* gene expression (Bohlman et al.1998).

Further evidence for separate jasmonate-dependent and salicylate-dependent defense-response pathways in Arabidopsis come from studies with *A.brassicicola*, *B.cinerea* and *P.parasitica* (Thomma et al. 1998). The *coi1* mutant showed enhanced susceptibility to virulent *A.brassicicola* and *B.cinerea* but not to *P.parasitica*, whereas *nahG* and *npr1* plants showed enhanced susceptibility to *P.parasitica* but not to the other two fungal pathogens (Thomma et al.1998).

Cross-talk between SA-dependent and JA-dependent signalling

Evidence that SA-dependent and JA-dependent signalling may influence each other by 'cross-talk' is accumulating (Bostock 1999, Malek & Dietrich 1999). Early evidence for negative cross-talk between SAR and the wound response in Arabidopsis came from pharmocological experiments where it was shown that SA inhibited wound-induced gene expression (Doares et al. 1995, Doherty et al. 1988). Furthermore, it was shown that SA prevented wound-induced gene expression in tomato by blocking synthesis of JA (Pena-Cortes et al. 1993). SA and JA also appear to have an antagonistic effect on *PR-1* gene expression in wounded tobacco leaves (Niki et al. 1998). It was found that JA induced the expression of basic *PR-1* and suppressed the expression of acidic *PR-1*, while SA was found to do the opposite (Niki et al. 1998).

Recent experiments have suggested an inverse relationship between SAR and resistance to insect herbivory (Felton et al. 1999). Transgenic plants silenced for PAL expression showed reduced SAR to TMV but enhanced grazing-induced resistance to larvae of *Heliothis virescens*. In contrast, transgenic plants overexpressing PAL exhibited enhanced SAR to TMV but larval resistance was reduced (Felton et al. 1999).

In a related experiment, separate tomato plants were infected with *Helicoverpa zea* larvae, the bacterial pathogen *Pst* or the fungal pathogen *Phytophthora infestans* , or plants were sprayed with BTH (Stout et al. 1999). The effects of these treatments on expression of defence genes and resistance to *Pst* or *H.zea* was determined (Stout et al. 1999). Inoculation with *Pst* increased resistance in the same plant to *H.zea* and *Pst.* Similarly, feeding by *H. zea* caused systemic resistance to both *H. zea* and *Pst.* In contrast, inoculation with *P. infestans* had no effect on resistance to *H.zea* (Stout et al. 1999). BTH treatment increased resistance to *Pst* but enhanced feeding of *H.zea. H. zea* feeding led to the systemic expression of *pin* mRNA, whereas *P. infestans* inoculation caused the accumulation of *PR* transcripts. *Pst* inoculation led to enhanced expression of both *pin* and *PR* genes (Stout et al. 1999). These results provide evidence for reciprocal induced resistance in tomato against multiple pathogens, and suggests that induced resistance to some pathogens may compromise resistance to others (Stout et al. 1999).

Analysis of Arabidopsis SAR mutants also provides evidence for interactions between SA-dependent and JA-dependent resistance. For example, the *cpr5* mutant displays constitutive expression of SA-dependent *PR-1* and the JA-dependent *PDF1.2*, which may indicate that the expression of these two genes is co-regulated (Bowling et al. 1997). This is consistant with the co-induction of both pathways in Arabidopsis by *Pst* inoculation (Maleck & Dietrich 1999). When the *cpr6* mutant was crossed with *nahG*, expression of the *PR-1* gene was significantly reduced, but *PDF1.2* expression was enhanced to levels higher than in *cpr6* alone (Clarke et al. 1998). This indicates that the elevated SA levels in *cpr6* may be suppressing *PDF1.2* expression. However, studies with the *ssi1* mutant suggest that SSI1 may function as a switch modulating cross-talk between the SA- dependent and JA-dependent signal transduction pathways (Shah et al. 1999). Evidence for this comes from suppression of *PDF1.2* expression in the *ssi1 npr1 nahG* triple mutant, which could be restored by spraying the plants with BTH (Shah et al. 1999). However, it is also possible that the *ssi1* mutant simply defines a common step in the signal transduction pathway, upstream of the branch point between SA-dependent and JA-dependent signal transduction. Although the *ssi1* mutation is dominant, triploid

plants containing one *ssi1* allele were phenotypically wild-type, indicating that the dominant nature of the *ssi1* mutation was due to haploinsufficiency i.e. that one wild-type copy is insufficient to rescue the defect caused by the *ssi1* mutation in diploid plants (Greenberg 2000). This implicates *SSI1* as a negative regulator of SA-dependent SAR (Greenberg 2000). In conclusion, some of the current evidence for cross-talk between SA-dependent signalling and JA-dependent signalling is some what contradictory, but further work in this area may help elucidate the complexity of the acquired resistance signal transduction network.

Other mutants with altered disease resistance

It is very likely that other signal transduction pathways, in addition to salicylate, ethylene and jasmonate dependent responses, are important in establishing SAR. Other mutant screens have been deployed in order to uncover further signal transduction pathways.

Phytoalexin-deficient mutants

In order to determine the role of camalexin, the predominant phytoalexin produced in Arabidopsis, *pad (p*hyto *a*lexin *d*eficient) mutants were isolated (Glazebrook et al. 1997b). Five complementation groups were identified. Mutations in *PAD1, PAD2* and *PAD4* caused enhanced susceptibility to *Psm*, while mutations in *PAD3* and *PAD4* did not (Glazebrook et al. 1997b). It was also shown that *PAD1, PAD2, PAD3* and *PAD4* are required for resistance to *P.parasitica* (Glazebrook et al. 1997b). In *pad4* plants inoculated with virulent *Psm*, SA levels, synthesis of camalexin, and *PR-1* transcript accumulation are all reduced (Zhou et al. 1998). Hence, *PAD4* may play an important role in acquired resistance. No such defects were seen after inoculation with *Psm (avrRpt2)* (Zhou et al. 1998). Treatment of *pad4* plants with SA partially reversed the camalexin deficiency and induced *PR-1* expression, suggesting that PAD4 operates upstream of SA accumulation in response to infection by virulent bacteria (Fig.1.3). *PAD4* may therefore participate in a positive regulatory loop that increases SA levels (Zhou et al. 1998). *PAD4* has been cloned recently and the predicted protein sequence displays similarity to triacyl glycerol lipases (Jirage et al. 1999).

The *pad3* mutant has also shown enhanced susceptibility to *A.brassicicola* (Thomma et al. 1999b), but not *B.cinerea* or *Erisphe orontii* (Thomma et al. 1999b, Reuber et al. 1998). Thus, PAD3 appears to be a key determinant in resistance to *A.brassicicola* (Thomma et al. 1999b). *PAD3* has also recently been isolated by map-based cloning and the predicted protein appears to be a cytochrome P450 monooxygenase, similar to an enzyme from maize that catalyses synthesis of an indole-derived metabolite (Zhou et al. 1999). *PAD3* expression is tightly correlated with camalexin synthesis, indicating that *PAD3* may encode an enzyme required for camalexin synthesis (Zhou et al. 1999). The *PR-1* and *PDF1.2* genes were not reduced in *A.brassicicola* infected *pad3* plants, indicating that camalexin production is controlled by a pathway that does not display cross-talk with SA-dependent and JA-dependent SAR (Thomma et al. 1999b).

Enhanced disease susceptibility mutants

In order to identify plant defence responses that limit pathogen attack, more *e*nhanced *d*isease *s*usceptability (*eds*) mutants that exhibit enhanced disease susceptibility to virulent *Psm* were identified (Glazebrook et al. 1996). At least 8 previously unidentified genes have been uncovered (Volko et al. 1998, Glazebrook et al.1997a). Apart from *eds4* and *eds5*, none of the *eds* mutants showed a significant alteration in the HR or SAR responses (Rogers & Ausabel 1997, Glazebrook et al. 1996), indicating that they define a new set of defence-related functions aimed at limiting the growth of virulent pathogens. *PR-1* expression and SA accumulation was reduced in *eds5* plants following *Psm* infection but camalexin production was unaffected (Rogers & Ausabel 1997). Thus, *EDS5* probably operates upstream of SA accumulation in a way that does not interfere with camalexin accumulation (Fig.1.3). Recent characterisation of the *eds4* mutant indicates that *EDS4* plays a role in SA-dependent SAR, as *PR-1* expression and SA accumulation following *Psm* inoculation was reduced and SAR was impaired (Gupta et al. 2000). Thus it has been proposed that *EDS4* acts in elevating SA following *Psm* inoculation, possibly by participation in an SA amplification loop (Fig.1.3) (Gupta et al. 2000). The *eds4* and *pad4* mutants also caused heightened expression of *PDF1.2* in response to rose

bengal and Me-JA, supporting the idea that SA accumulation interferes with JA-dependent signalling (Gupta et al. 2000).

Two *sid* (*s*alicylic acid *i*nduction *d*eficient) mutants were isolated in a screen designed to quantify SA accumulation in each individual mutant (Nawrath et al. 1999). Both *sid* mutants did not accumulate SA in response to *Pst* (*avrRpt2*), were susceptible to both virulent and avirulent *Pst* and *P.parasitica* and showed reduced expression of *PR-1* but not *PR-2* and *PR-5* in response to *Pst (avrRpt2)* (Nawrath et al. 1999). In addition, both mutants were found to have a blockage in SA biosynthesis (Nawrath et al. 1999), indicating that they operated upstream of SA in the SAR signal transduction network (Fig.1.3). The *sid1* mutant was shown to be allelic to *eds5*, but *sid2* defines a previously unidentified gene involved in SA biosynthesis and SAR signal transduction (Nawrath et al. 1999) (Fig.1.3).

Enhanced disease resistance mutants.

The *edr1* (*e*nhanced *d*isease *r*esistance) mutant displays enhanced resistance to *Pst* and *Erysiphe cichoracearum*, but does not constitutively express the *PR-1, PR-2* and *PR-5* SAR marker genes (Frye & Innes 1998). Thus *edr1* describes a novel mutant class. *E. cichoracearum* conidia germinate and formed extensive hyphae on *edr1* plants, but subsequent conidiophore production and sporulation is drastically reduced in comparison to wild-type plants, indicating that *EDR1* can be considered to be a 'late-acting' gene in resistance to powdery mildew (Frye & Innes 1998). More recently, four additional *E. cichoracearum* resistant mutants were isolated (Vogel & Sommerville 2000). The *pmr* (*p*owdery *m*ildew *r*esistant) mutants did not display constitutive expression of *PR-1* or *PDF1.2* and, unlike *edr1*, were not more resistant to *Pst* (Vogel & Sommerville 2000). This indicates that the *pmr* mutants define a different disease resistance class in comparison to *edr1*.

1.6. Aims of the project

The overall aim of the project is to further investigate the mechanism of the signal transduction network leading to the establishment of acquired resistance in Arabidopsis. The pathway beginning with the interaction between the *avrB* and *RPM1* gene products, and culminating in the local expression of *PR-1* was relevant to this project. Transgenic Arabidopsis plants expressing the luciferase reporter gene under the control of the *PR-1a* promoter (designated *PR-1a:luc)* were generated as a tool to study *PR-1a* gene expression. Real-time *PR-1a:luc* expression following inoculation with an avirulent bacterial pathogen could be monitored by detection of luc activity in an ultra low-light imaging camera. A genetic approach was taken in order to further study this pathway and mutants were generated by chemical mutagenesis. Potential mutants were identified by visualising abnormal luc activity. A group of potential mutants were chosen for characterisation and one, *cir1* (*c*onstitutively *i*nduced *r*esistance *1*) was confirmed as a true disease resistance mutant.

The following chapter (Chapter 2) details the materials and methods used in this study.

Chapter 3 outlines the production of the *PR-1a:luc* transgenic lines and describes the pattern of luc activity produced following inoculation with the avirulent bacterial pathogen, *Pst* DC3000 *(avrB)*. Mutants were generated in homozygous *PR-1a:luc* plants, and M2 mutant plants were screened for abnormal luc activity following inoculation with *Pst* DC3000 *(avrB)*. Several classes of potential mutants were uncovered.

Chapter 4 discusses the characterisation of two groups of these potential mutants, *neb* (*n*o *e*xpression of *b*ioluminescence) with a block upstream of SA in the acquired resistance signal transduction pathway, and *ceb* mutants, which showed constitutive expression of *PR-1a:luc*. Luminometer assays were conducted in order to quantify *PR-1a:luc* expression and Northern blot analysis was used to study expression of

various defence-related genes. Disease resistance assays to virulent pathogens (*Pst*DC3000, *P. parasitica* Noco2 and *F. oxysporum* f.sp. *matthiolae*) were performed. One of the *ceb* mutants, re-named *cir1,* was selected as *bona fide* SAR mutant.

Chapter 5 shows further characterisation of *cir1*. Genetic analysis was conducted by crossing *cir1* with various other Arabidopsis lines, in order to determine the segregation and allelism of *CIR1*. The relationship of *cir1* to SA, JA and ethylene was also investigated by crossing *cir1* to SA-, JA- and ethylene-insensitive mutants. The position of the *CIR1* gene in the Arabidopsis genome was determined by mapping with SSLP and CAPS markers.

Finally, Chapter 6 discusses the results with reference to current ideas about the SAR signalling network in Arabidopsis, and the potential for its application in the development of better crops.

Chapter Two

Materials and Methods

Unless otherwise stated, all chemicals were purchased from Sigma (Sigma-Aldrich, UK).

2.1. Growth of *Arabidopsis thaliana*

*Arabidopsis thaliana (*Arabidopsis) seeds of ecotype Columbia (Col-0) and Landsberg erecta (L*er*) were used. All Arabidopsis transgenic lines and mutant strains used were in a Col-0 background and are outlined in Table 2.1. Most seeds were obtained from the Nottingham Arabidopsis Stock Centre (NASC). Seeds were placed on potting medium consisting of peat moss, vermiculite and sand (4:1:1), and allowed to vernalise for 48 hours at 4°C after which they were transferred to 20°C. Plants were placed 4 to a pot, were watered by sub-irrigation and were fertilised once a week with Phosphogen®. In order to promote the growth of healthy leaves, plants were placed under short day length conditions (10 hours light, 14 hours dark) at 20°C, otherwise plants were placed in the transgenic greenhouse under longer day length conditions (16 hours light, 8 hours dark).

2.2. Growth of *Pseudomonas syringae* pv *tomato* DC3000 (*avrB*) and inoculation of plants

Pseudomonas syringae pv *tomato* DC3000 (*avrB*) (*Pst*DC3000 (*avrB*)) was grown on Kings broth (King et al. 1954) supplemented with 50 $mg.l^{-1}$ rifampicin and 50 $mg.l^{-1}$ kanamycin. Liquid cultures were grown on a shaker at 30°C, and cells were harvested at OD_{600} equal to 0.2 (the equivalent of 10^6 colony forming units per cm^{-2} ($cfu.cm^{-2}$). Cells were pelleted by centrifugation and re-suspended for plant inoculation in 10 mM $MgCl_2$. For inoculations, 6 µl of the *Pst*DC3000 (*avrB*) solution were forced under the abaxial epidermis using a 1 ml syringe. Successful inoculations were visualised by the appearance of a watery area under the epidermis.

Table 2.1. Arabidopsis transgenic lines and mutant strains.

Strains	Phenotype	Reference	Source
Col-0	wild-type		NASC
L*er*	wild-type		NASC
PR-1a:luc	*PR-1a:luc* transgenic	Thomson & Loake	Thomson & Loake
nahG	Salicylate hydroxylase transgenic	Lawton et al. 1995	Novartis, USA
npr1-1	SAR insensitive	Cao et al. 1995	Dong, Duke University
ein2-1	Ethylene insensitive	Guzman & Ecker 1990	NASC
etr1-1	Ethylene insensitive	Bleeker et al. 1988	NASC
jar1-1	Jasmonate insensitive	Staswick et al. 1992	Staswick, University of Nebraska
coi1-1	Jasmonate insensitive	Feys et al. 1994	Turner, University of East Anglia
cpr1-1	Constitutive SAR	Bowling et al. 1994	Dong, Duke University
cpr5-1	Constitutive SAR	Bowling et al. 1997	Dong, Duke University

2.3. Treatment of plants with salicylic acid or methyl jasmonate

A 10 mM stock solution of salicylic acid (SA) was made up in 10 mM sodium phosphate buffer, pH 7.2 (Sambrook et al. 1989). A 1:10 dilution, containing 0.001% (v/v) Silwet (Ambersil Ltd, UK) and 0.001% (v/v) triton-X, was used to paint the leaves. These leaves were imaged 24 hours later for *PR-1a:luc* expression using the ultra-low light imaging camera or harvested for RNA extraction.

A 20mM methyl jasmonate (Me-JA, Aldrich) stock solution was prepared in 0.1% (v/v) ethanol. A 1:200 dilution, containing 0.001% (v/v) Silwet (Union Carbide) and 0.001% (v/v) triton-X, was used to paint the leaves which were harvested after 24 hours for RNA extraction.

2.4. *In vitro* selection of Arabidopsis seedlings

Arabidopsis seedlings were tested *in vitro* by addition of various compounds to the tissue culture medium. *PR-1a:luc* transgenic seeds were tested for kanamycin resistance, *nahG* transgenic seeds were tested for the appearance of brown roots on MS medium supplemented with 0.5mM SA (Bowling et al. 1994), *jar1* seedlings were tested for insensitivity to 10μM Me-JA (Staswick et al. 1992) and *ein2.1* and *etr1* mutants were selected for insensitivity to 10μM 1-aminocyclopropane-1-carboxylic acid (ACC) in the dark (Oh et al. 1997). In all cases, approximately 1000 transgenic Arabidopsis seed (20 mg) were sterilised for 20 minutes in 10 % (v/v) bleach containing 10 μl of Triton-X. Seeds were washed four times with sterile distilled water. Seeds were placed at 4°C for 2 days so vernalisation could take place. Seeds were re-suspended in 0.1% (w/v) agarose (1 ml used per 1000 seed) and plated out on MS selection plates (1 X MS salts (M5519), 0.3% (w/v) sucrose, 8g.l^{-1} agar, 200 mg.l^{-1} cefotaxime and 20 mg.l^{-1} Rovral, Rhone-Poulenc Ltd). For transgenic Col-0, 50 mg.l^{-1} kanamycin was used. After drying, plates were placed in the growth room (20°C, continuous light) for 7-10 days. Seedlings were scored for the appropriate phenotype under test and transferred to soil.

2.5. Viewing *PR-1a:luc* expression using the ultra-low light imaging camera

Luciferin (Biosynth AG) is the substrate for the firefly luciferase gene. Leaves, still attached to the plant, were painted *in situ* with 1 mM luciferin in a 100 mM Na-citrate buffer containing 0.001% (v/v) Silwet and 0.001% triton-X (v/v) (Ow et al. 1996). Following this, plants were placed in the dark for 30 minutes in order to allow the luciferin to dry and to minimise background bioluminescence.

In order to make imaging more easy during the mutant screen, leaves from four plants were excised and imaged simultaneously by placing the leaves on the same plate. In other cases the entire plant was imaged. Imaging was performed using a Berthold Luminograph (EG&G Wallac, Milton Keynes, UK). The Luminograph consists of an ultra-low light imaging camera attached to a dark box and a computer.

Leaves or plants were placed in the dark box for imaging. Bioluminescence images emitted from the leaves were collected for a 10 second accumulation period and integrated over a 5 second period using the Luminograph software. The image intensifier was set to 100% and gamma, flat-field and defect corrections were performed on the accumulated images in all cases. Images, saved as TIF files, were exported from the Luminograph software and processed using the Confocal Assistant (Biorad) and Adobe Photoshop software.

2.6. Luminometer assay

Luciferase activity was measured in the Berthold MicroLumat LB96P microwell plate luminometer (EG&G Wallac, Milton Keynes, UK). Crude protein extractions were made by grinding a leaf in one ml of lysis buffer (0.1M sodium phosphate pH 7.2, 5 mM DTT) (Doelling & Pikaard et al. 1993). The liquid was removed and centifuged (12000 rpm, 5 minutes). The supernatant was used directly to measure total protein content, and was diluted 2-fold with lysis buffer to assay for luciferase activity. Total protein content was measured using the Bradford micro-assay (Sigma) using bovine serum albumin (BSA) as the protein standard. Luciferase assays were performed by mixing 100 µl of assay buffer (60 mM Tris-HCl pH 8.0, 20 mM $MgCl_2$, 20 mM DTT, 2 mM EDTA, 2 mM ATP) (Doelling & Pikaard et al. 1993) with 100 µl of the protein extract. In another tube, 2 X assay buffer (without ATP) was mixed with an equal volume of 2 mM luciferin. Background luminescence for each sample was determined for 20 seconds prior to luciferin injection. Luciferase activity was measured in the 20 seconds following injection of the luciferin sample (100µl) into the protein sample. After background readings were subtracted, the luciferase activity for each sample was calculated as RLU per microgram of extractable protein.

2.7. RNA Gel Blot analysis

Total RNA was extracted from Arabidopsis leaves harvested from 5-week old plants using the guanadinium thiocyanate (GTC) phenol chloroform extraction method.

Leaf tissue (approximately 0.1g) was ground in liquid nitrogen using a pestle and mortar, poured into a 1.5 ml eppendorf and 0.45 ml GTC solution (4M guanadinium thiocyanate, 25 mM sodium citrate, 0.5% (w/v) sarcosyl, 0.1 M ß mercaptoethanol) was added. Following mixing by vortexing for 30s, 0.05 ml 2 M sodium acetate pH4.0, 0.45 ml phenol and 0.1 ml chloroform:iso-amylalcohol (49:1) were added. After further mixing, the tubes were placed on ice for 15 min. The samples were centrifuged at 10 000 rpm for 20 min, the supernatant was removed carefully from each tube and transferred to a new tube. An equal volume of isopropanol was added to each tube, mixed and left at -20°C for at least 2 hours or overnight in order to precipitate the RNA. After incubation, the RNA was recovered by centrifugation (10000 rpm, 20 min). The pellet was re-dissolved in 0.15 ml GTC solution, and re-precipitated by the addition of 0.15ml isopropanol and storage at -20°C for 1 hour. Following centrifugation (10 000 rpm, 20 min), the RNA pellet was washed twice in 70% ethanol, dried and dissolved in 100 µl DEPC-treated water. The absorbance of each sample was measured at 260 nm, and used to calculate the concentration of RNA. Samples (10 µg) were separated on formaldehyde-agarose gels (Sambrook et al. 1989), transferred to a Hybond™-N hybidization membrane according to the instructions of the supplier (Amersham Lifesciences) and hybridized with the relevant probes (described below). Dextran sulphate (10% w/v) was included in the pre-hybridization / hybridization solution in order to allow for efficient binding of the probe (Sambrook et al. 1989). Blots were washed twice for 30 min each at 65°C in 4 X SSC, 1% (w/v) SDS, which was followed by two washes at 65°C in 4 X SSC, 0.5% (w/v) SDS. Blots were exposed to X-Omat-AR™ imaging film (Kodak) for an appropriate time period. Blots were stripped by incubation in boiling 0.1% (w/v) SDS and washing in 0.5 X SSC for 30 min at room temperature, before hybridization with a subsequent probe (Sambrook et al. 1989).

Probes were prepared by amplification of appropriate sequences using PCR and directly purified using a kit (Promega). The expected PCR fragment size was verified by gel electrophoresis. Alternatively, probes were generated from plasmids by digestion with relevant restriction enzymes, identified by gel electrophoresis, and purified from the gel by freeze-thaw extraction. Sequences for the PCR primers,

templates used and the reference for each probe are shown in Table 2.2. Probes were labelled with α-^{32}P-dCTP by random priming using the Prime-a-Gene® labelling system (Promega). In order to identify lane-to-lane variations in the amount of RNA added and thus facilitate comparisons between lanes, each blot was probed with the ribosomal 18S (r18) probe.

Table2.2. Reagents used in the generation of probes.

Gene	Template	Forward primer	Reverse primer	RE	Probe (kb)
PR-1	TA-PR1 [a]	CTgCAgACTCATACACTCTgg [a]	TATgTACgTgTgTATgCATgATC [a]	-	0.3
PR-2	pBluescript-PR2 [a]	T7	T3	-	1.2
PR-5	pBluescript-PR5 [a]	T7	T3	-	1.0
GST1	GST1 [b]	GgTTCTTTAAgTgAATCTCAAAC [b]	CAAgACTCATTATCgAAgATTAC [b]	-	1.0
PDF1.2	genomic DNA	TCATggCTAAgTTTgCTTCC [c]	AATACACACgATTTAgCACC [c]	-	0.3
Thi2.1	pUC19-Thi2.1 [d]	-	-	EcoR1	0.7
ACS2	genomic DNA	CgAggAAgAACATTCACCTA [e]	CgATCTCTCAgTAGATgTCT [e]	-	0.45
LOX2	pZL1-LOX2 [f]	-	-	Sal1-Not1	2.0
r18	pBluescript-18S [g]	T7	T3	-	1.5

[a] Uknes et al. 1992. *PR-1* was initially isolated by PCR from an Arabidopsis cDNA library with the forward and reverse primers, and cloned into the TA vector (Thomson & Loake, unpublished).
[b] Sharma et al. 1996, Yang et al. 1998
[c] Penninckx et al. 1996
[d] Epple et al. 1995
[e] Liang et al. 1992 (GenBank accession no.: M95594)
[f] Obtained from AIMS stock centre, stock no. 106C8T7 (GenBank accession no.: T22547)
[g] Pruitt & Meyerowitz 1986

2.8. Virulent disease resistance assays

2.8.1. Resistance to *Pseudomonas syringae* pv *tomato* DC3000

P.syringae pv *tomato* DC3000 (*Pst*DC3000) (Whalen et al. 1991) was grown in King's broth liquid media (King et al. 1954) supplemented with 50 mg.l^{-1} rifampicin. Four week old soil-grown plants were infected with a *Pst* DC3000 suspension (OD_{600} = 0.0002) in 10 mM $MgCl_2$ by completely infiltrating the abaxial side of the leaf with a 1 ml syringe (Cao et al. 1994). Three leaves per plant, and three plants per line were infiltrated. After three days, plants were inspected for development of symptoms. Leaves were also harvested at this time point for analysis of bacterial

growth. Leaf discs of uniform size ($0.5cm^2$) were made from the leaf samples using a cork borer. Three leaf discs from each plant were ground in 990 µl 10 mM $MgCl_2$ in a pestle and mortar. Serial dilutions were made from the resulting bacterial suspension, and 100 µl of each dilution was used to inoculate King's B medium plates containing 50 $mg.l^{-1}$ rifampicin. The plates were incubated at 30°C for 2 days, and the number of bacterial colonies for each sample was recorded. Three days prior to inoculation with *Pst*DC3000, some Col-0 plants were induced to activate SAR by spraying with 1 mM SA. Bacterial counts were statistically analysed using the Mann-Whitney test (Mini-tab version 12).

2.8.2. Resistance to *P.parasitica* Noco2

The *Peronospora parasitica* Noco2 oomycete pathogen (Parker et al. 1993) was maintained in Col-0 seedlings grown under humid conditions and conidiospores were transferred to new seedlings weekly by dusting infected seedlings onto healthy seedlings. For the *P.parasitica* disease resistance assays, conidiospores were harvested by vortexing infected seedlings in water. The spore concentration was determined using a haemocytometer, and adjusted to $1X10^5$ spores per ml. Four-week old plants grown under short day conditions (10 hours light, 14 hours dark) were sprayed with the conidiospore solution and maintained under humid conditions. Results were taken after 10 days, and infected plants were scored for the extent of downy mildew growth (visualised as condiophore growth) based on Bowling et al. (1997). Scoring was as follows: 0=no infection, 1=less than 25% of one leaf with conidiophore growth, 2=25 to 50% of one or two leaves covered with condiophores, 3=25 to 50 % of three or four leaves covered with conidiophore growth, 4=25 to 50% of all leaves covered with condiophore growth, 5=all leaves covered with conidiophore growth. Plants in different replicates were assigned a disease index as follows: $D.I.=\sum i \times j/n$, where i=infection class, j=the number of plants scored for that infection class and n=the total number of plants in the replicate (based on Epple et al. 1997a).

2.8.3. Resistance to *Fusarium oxysporum* f.sp. *matthiolae*

Fusarium oxysporum f.sp. *matthiolae* (Mauch-Mani & Slusarenko 1994) was maintained on potato dextrose agar plates at room termperature. Cultures were sub-cultured onto fresh medium every two to three weeks. For inoculation of plants, spores were harvested by gently rubbing the mycelial growth on each plate and re-suspension in 10 ml sterile water. Spores were separated from mycelia by centrifugation (4000 rpm, 15 minutes) and the resulting pellet, containing the spores, was re-suspended in 5 ml sterile water. Spore concentration was determined using a haemocytometer, and adjusted to $1X10^5$ spores per ml (Epple et al. 1997a). Four-week old plants grown under short day conditions (10 hours light, 14 hours dark) were sprayed with the spore solution and maintained under humid conditions. Three replicates, containing four plants each, were inoculated. Results were taken after 8 days and infected plants were scored for the number of necrotic leaves. Symptom development was expressed as the percentage of nectrotic leaves per plant. Scores were statistically analysed using the Mann-Whitney test (Mini-tab version 12).

2.9. Determination of SA levels

SA measurements were performed with leaves harvested from 5-week old plants according to Bowling and co-workers (1994). Frozen leaf tissue samples (1g) were extracted with methanol, dried down and re-suspended in 0.01M H_2SO_4, and free SA levels were analysed using an HPLC method.

2.10. Determination of ethylene evolution

Ethylene measurements were performed using 5-week old plants according to Iannetta and co-workers (1999). Six plants per line were enclosed individually in glass jars and evolved ethylene was collected over a 24 hour period. One ml of head space was removed for ethylene determination by gas chromatography. After incubation, the fresh weight of each plant was determined.

2.11. Trypan blue staining

Leaf samples were boiled for 2 minutes in a lactic acid-phenol-trypan blue solution (2.5mg.ml^{-1} trypan blue, 25% (w/v) lactic acid, 23% water saturated phenol, 25% glycerol and water) (Bowling et al. 1997).. After cooling for one hour, the lactic-acid-phenol-trypan blue solution was replaced with a chloral hydrate solution (25g in 10 ml water) for de-staining. After 24 hours, the chloral hydrate solution was removed and the samples were equilibrated in 70% (v/v) glycerol and mounted onto microscope slides. Stained leaves were viewed for micro-lesions, or small areas of dead tissue, using the Leica Wild M3C microscope.

2.12. Genetic analysis

Crosses were performed by dissecting and emasculating unopened buds two days prior to anthesis, and using the pistils as recipients for pollen from open donor flowers (Koorneef & Stam 1992). In order to determine the dominant/recessive nature of the mutation in the *ceb* candidate mutant lines, pollen from each *ceb* plant was used to pollinate separate wild-type *PR-1a:luc* transgenic plants. The number of segregation groups amongst the *ceb* candidate mutants was determined by crossing the *ceb* plants against each other (Koorneef & Stam 1992). F1 and F2 five-week old plants were tested for constitutive *PR-1a:luc* expression using the ultra low-light imaging camera.

Pollen from homozygous *cir1* plants was used to pollinate *nahG, npr1, jar1, ein2.1* and *etr1* plants. As *jar1, ein2.1* and *etr1* do not contain the kanamycin resistance gene, F1 progeny were tested for kanamycin resistance in order to indicate that the cross had been successful. F1 seeds were germinated on MS medium supplemented with kanamycin as described above. F2 five-week old plants from all crosses were tested for constitutive *PR-1a:luc* expression using the ultra low-light imaging camera. All F2 plants producing constitutive luc activity were allowed to set seed and were tested further. F3 *cir1:nahG* seeds were tested for brown roots in germinating seedlings placed on MS medium supplemented with 0.5 mM SA as

described above. F2 *cir1:npr1* plants were tested for the presence of the *npr1* mutation using a CAPS PCR marker. The *npr1* mutation abolishes a NLaIII restriction site in the PCR fragment generated by this marker, thus providing a method for distinguishing *npr1* from wild-type (Clarke et al. 1998, Cao et al. 1997). F2 *cir1:jar1* plants were identified by their small, dark green leaves typical of *jar1* (Staswick et al. 1992) and constitutive *PR-1a:luc* expression. F3 *cir1:jar1* plants were tested for insensitivity to Me-JA as described above. F2 progeny from the *ein2.1 X cir1* and *etr1 X cir1* crosses, expressing *PR-1a:luc* constitutively, were identified. These plants were allowed to set seed and the F3 progeny from these plants were tested for ACC insensitivity as described above. F3 *cir1:ein2.1* and *cir1:etr1* were thus identified.

2.13. Mapping using SSLP and CAPS markers

In order to generate an F2 mapping population, *cir1* was crossed to L*er*. F2 plants expressing constitutive luc activity were selected for mapping. Mapping was performed using the co-dominant cleaved amplified polymorphic sequences (CAPS) protocol described by Konieczny & Ausabel (1993) and the single sequence-length polymorphisms (SSLPs) protocol described by Bell & Ecker (1994). Both protocols are based on PCR of polymorphic genomic DNA sequences between Col-0 and L*er*. The PCR primers used for both types of markers and the restriction enzymes used for the CAPS protocol are outlined at The Arabidopsis Information Resource (TAIR) (http://www.arabidopsis.org/). The markers used in the present project are listed in Table 2.3.

Genomic DNA was extracted from leaf tissue using a small-scale method (Edwards et al. 1991). Approximately 1 cm^2 of leaf tissue was ground in 0.5ml DNA extraction buffer with carborandum. Samples were incubated at 60°C for 10 min followed by extraction with an equal volume of chloroform:isoamyl alcohol (24:1). After centrifugation (10 000rpm, 10 min), the DNA was precipitated from the resulting supernatant by the addition of 2X volume ethanol and 1/10 volume 3M sodium acetate, pH 5.2. After a further centrifugation step (10 000rpm, 10min), the

DNA pellet was washed in 70% (v/v) ethanol twice and finally re-suspended in 50 µl TE (Sambrook et al. 1989). RNase (10 µg) was added and samples were incubated for one hour at room temperature in order to eliminate contaminating RNA species.

Primers for the different markers are listed in Table 2.2 and all primers were manufactured by Amersham Pharmacia. PCR reactions were performed using 10X buffer, magnesium chloride and *Taq* polymerase (Promega) and deoxynucleotides from Boehringer (Roche Molecular Biochemicals). The PCR reactions were carried out in 10 µl volume containing 1µl of the DNA miniprep (approximately 10 ng DNA), 5 pmol of each primer, 200 µM each of four deoxynucleotides, 2mM magnesium chloride and 1 unit of *Taq* polymerase. PCR reactions were carried out in either a Hybaid Omn-E thermal cycler or a Peltier 200 thermal cycler. For the CAPS markers, conditions were as follows: 30s at 94°C, primer annealing for 30s at 56°C, primer elongation for 3 min at 72°C. This cycle was repeated 50 times. For the SSLPs markers, conditions were as follows: 30s at 94°C, primer annealing for 30s at 56°C, primer elongation for 1 min. This cycle was repeated 30 times. Products from the CAPS PCR reactions were digested with the relevant restriction enzymes listed in Table 2.2. All digests were performed at 37°C with restriction enzymes purchased from Promega except for BsaB1 which was purchased from New England Biolabs. Digests with BsaB1 were performed at 60°C. A restriction enzyme mix (10 µl) containing 2 µl of restriction enzyme buffer and 1.5 units of restriction enzyme were added to each 10 µl CAPS PCR tube and samples were incubated for 2 hours at the optimal temperature for the particular enzyme. For both the SSLP PCR products and the CAPS digestion products, 5 µl of loading dye (Sambrook et al. 1989) was added to each tube prior to electrophoresis. Agarose gel concentrations optimised for each marker are listed in Table 2.2. Col-0 and L*er* samples were included on each gel as controls and run in adjacent bands in order to visualise polymorphisms. The 1kb DNA ladder (Gibco-BRL) was also included on each gel in order to determine the size of different PCR or restriction digestion products.

Table 2.3. List of CAPS and SSLP markers used in mapping experiments.

Restriction enzymes required for analysis of CAPS markers, gel conditions for analysis of PCR products, observed PCR products polymorphic between Col-0 and L*er* and primer sequences for the markers are shown.

						Observed band size (kb)				
Chr	cM [a]	Name	Type	Enzyme	Gel	Fragment	Col-0	Ler	Forward primer seq	Reverse primer seq
1	9.3	nga63	SSLP		2% 2h,82V		0.111	0.089	ACCCAAgTgATCgCCACC	AACCAAggCACAgAAgCg
1	46	AthSO392	SSLP		3% MP* 4.5h ,45V		0.142	0.156	TTTggAgTTAgACACggATCTg	gTTgATCgCAgCTTgATAAgC
1	100	nga128	SSLP		2% 2h,82V		0.18	0.19	ggTCTgTTgATGTCgTAAgTCg	ATCTTgAAACCCTTTAgggAggg
2	30	THY1	CAPS	RSA1	2% 2h,82V	0.81	0.6/0.1	0.6/0.5/0.4/0.3/0.1	ggCgACCTTggACCTgTATACg	AACCgCCATTTTCATTTCTATC
2	73	nga168	SSLP		3% MP* 4.5h ,45V		0.151	0.135	gAggACATgTATAggAgCCTCg	TCgTCTACTgCACTgCCG
3	30	ArLIM15	CAPS	ECoR1	1% 2h,82V	0.5	0.45/ 0.05	0.5	gCCAgTTTTTTCCTgCACATCAATC	TgCTgCTTTATTTTgTCgCgATgTT
3	75	nga707	SSLP		3% MP* 4.5h ,45V		0.132	0.128	TGAATgCGTCCAgTgAgAAg	CTCTCTgCCTCTCGCTgg
4	17.7	GA1	CAPS	BSaB1	1% 2h, 82V	1.2	1.2/0.8/0.4	1.2	AAgCTTCgAACTCAAggTTC	CCggAgAATCgTACggTAC
4	26.5	nga8	SSLP		1% 2h,82V		0.154	0.198	gAgggCAAATCTTTATTTCgg	TggCTTTCgTTTATAAACATCC
4	29.6	nga1111	SSLP		4% MP* 6.5h, 45V		0.148	0.154	AGTTCCAgATTgAgCTTTGAgC	gggTTCggTTACAATCgTgT
4	57.6	g4539	CAPS	HindIII	1% 2h, 82V	0.6	0.6	0.48/0.12	ggTCATCCgTTCCCAggTAAAg	ggACgTAgAATCTgAgAgCTC
4	63.1	AG	CAPS	Xba1	2% 2h,82V	1.366	1.366	1.073/0.293	CAAACACCATTTAATCTTgACA	CAACAggTTTCTTCTTCTTCTC
4	75.7	RPS2	CAPS	Sau3A	2% 2h,82V	0.785	0.18/0.605	0.18/0.251/0.354	CTCAgAgTCTTggACTTgTCg	TTCgACggATggACTCTCgTg
4	102	nga1107	SSLP		2% 2h,82V		0.15	0.14	gCgAAAAAACAAAAAAATCCA	CgACgAATCgACAgAATTAgg
5	26	nga151	SSLP		2% 2h,82V		0.15	0.12	gTTTTgggAAgTTTTgCTgg	CAgTCTAAAAgCgAgAgTATgATg
5	62	AtSO262	SSLP		3% MP* 4.5h ,45V		0.145	0.159	CTCCACCAATCATgATgCAAATg	TgATgTTgATggAgATggTCA
5	103	AthSO191	SSLP		3% MP* 4.5h ,45V		0.148	0.156	TgATgTTgATggAgATggTCA	CTCCACCAATCATgCAAATg

[a]cM, centimorgans. Position of the marker on the genetic map (Lister and Dean 1993).

* MP, MetaPhor agarose (FMC Bioproducts)

Chapter Three

Screening for Arabidopsis SAR mutants

3.1. Introduction

A genetic approach was undertaken in this project in order to study SAR. This entailed isolating *Arabidopsis thaliana* (Arabidopsis) mutants. In order to allow for easy isolation of candidate mutants, mutations were generated in a transgenic Arabidopsis line expressing the luciferase (*luc*) reporter gene under the control of the *PR-1a* reporter gene. Transgenic plants thus produced luciferase (luc) activity during the establishment of SAR and it was possible to identify candidate mutants with changes in *PR-1a:luc* expression in comparison to transgenic plants.

3.2. Production of *PR-1a:luc* transgenic Arabidopsis plants

Arabidopsis ecotype Columbia (Col-0) plants were transformed with a construct containing the -903bp *PR-1a* promoter from tobacco (Payne et al. 1988) fused to the firefly luciferase (*luc*) marker gene (Promega) and the *ocs* terminator from *Agrobacterium tumefaciens* (Fig.3.1) (Thomson & Loake, unpublished results). This construct also contained the kanamycin resistant gene, so transgenic seedlings could be selected for kanamycin resistance. Sixteen individual kanamycin resistant *PR-1a:luc* transgenic seedlings were identified (Thomson & Loake, unpublished results). All 16 lines were tested for luc activity following inoculation with *Pseudomonas syringae* pv. tomato DC3000 expressing the *avrB* avirulence gene (*Pst*DC3000 (*avrB*)) (Innes et al. 1993). The *avrB* avirulence gene was isolated from *Pseudomonas syringae* pv. *glycinea* Race 4, a pathogen of soybean, and transferred to the virulent Arabidopsis bacterial pathogen *Pst*DC3000 (Innes et al. 1993). Inoculation of Arabidopsis ecotype Col-0 plants with the *Pst*DC3000 (*avrB*) strain resulted in visualisation of a HR 24 hours after inoculation (Innes et al. 1993). The *luc* gene encodes an enzyme that catalyzes the ATP-dependent oxidation of luciferin, producing light at 465 nm (Ow et al. 1986). Inoculated leaves were painted with luciferin and luc activity in real time was imaged by means of an ultra low-light

imaging camera. *PR-1a:luc* transgenic plants expressed light around the HR lesion following inoculation with *Pst*DC3000 (*avrB*) (Thomson & Loake, unpublished results). A highly inducible line with low background luc activity was chosen for further study (Thomson & Loake, unpublished results). Plants from this line were allowed to self-fertilise and T2 seed was tested *in vitro* for kanamycin resistance. Over 1000 F2 seedlings were scored and it was found that the segregation ratio of kanamycin resistant seedlings to kanamycin susceptible seedlings was 3:1. This indicated that the *PR-1a:luc* transformation cassette (Fig.3.1) had integrated into a single position in the Arabidopsis genome (Thomson & Loake, unpublished results). T2 plants were allowed to self-fertilise and T3 seeds from individual T2 plants were collected. T3 seedlings were scored for kanamycin resistance and T2 plants showing 100% kanamycin resistance in their T3 progeny were identified as being homozygous for the *PR-1a:luc* transformation cassette. Homozygous *PR-1a:luc* seeds were used for all further studies.

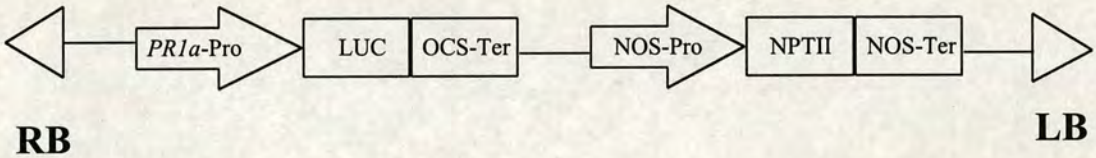

Fig.3.1. The transformation cassette used to produce *PR-1a:luc* transgenic plants.

LB-left border, LUC-luciferase gene, NPTII- neomycin phosphotransferase (encoding kanamycin resistance), NOS- nopaline synthase, OCS- octapine synthase, Pro- promoter, RB- right border, Ter-terminator.

3.3. Expression of *PR-1a:luc* in transgenic plants.

In order to use the *PR-1a:luc* transgenic plants to generate mutations and screen for candidate mutants expressing abnormal luc activity during SAR, it was first necessary to describe the pattern of luc activity produced by the wild type *PR-1a:luc* plants. Two methods (outlined in Chapter 2) were used to investigate the induction

of luc activity in *PR-1a:luc* plants: imaging with the ultra low-light camera and a luminometer assay.

SAR is initiated by inoculation of a resistant plant with an avirulent pathogen and is associated with SA accumulation and SA-dependent expression of *PR-1* (Delaney 1997, Ryals et al. 1996, Ryals et al. 1994). Thus, the extent of luc activity, as an indication of *PR-1* expression, was investigated in *PR-1a:luc* transgenic plants in response to inoculation with avirulent bacterial pathogens or treatment with SA. The luminometer assay was used in order to quantify luc activity. *PR-1a:luc* plants were inoculated with *Pst*DC3000 (*avrB*), *Pst*DC3000 *(avrRpt2),* $MgCl_2$ (the control) or painted with 1mM SA. Infected leaves were harvested 3 days (72 hours) after inoculation and SA-treated leaves were harvested 24 hours after application. Extracts were made and the luc activity of the extract was determined. Luciferin was injected individually into each well of the luminograph plate and the light produced in the 20 seconds following injection was recorded. When luciferase comes into contact with luciferin, a flash of light is produced (Ow et al. 1986). It was decided to record the amount of light produced for 20 seconds as both the flash of light and the stable linear production of luciferase activity would be recorded. Luc activity was calculated as the relative light units (RLU) per microgram of extractable protein produced during the 20 seconds following luciferin injection. Results obtained are outlined in Fig.3.2.

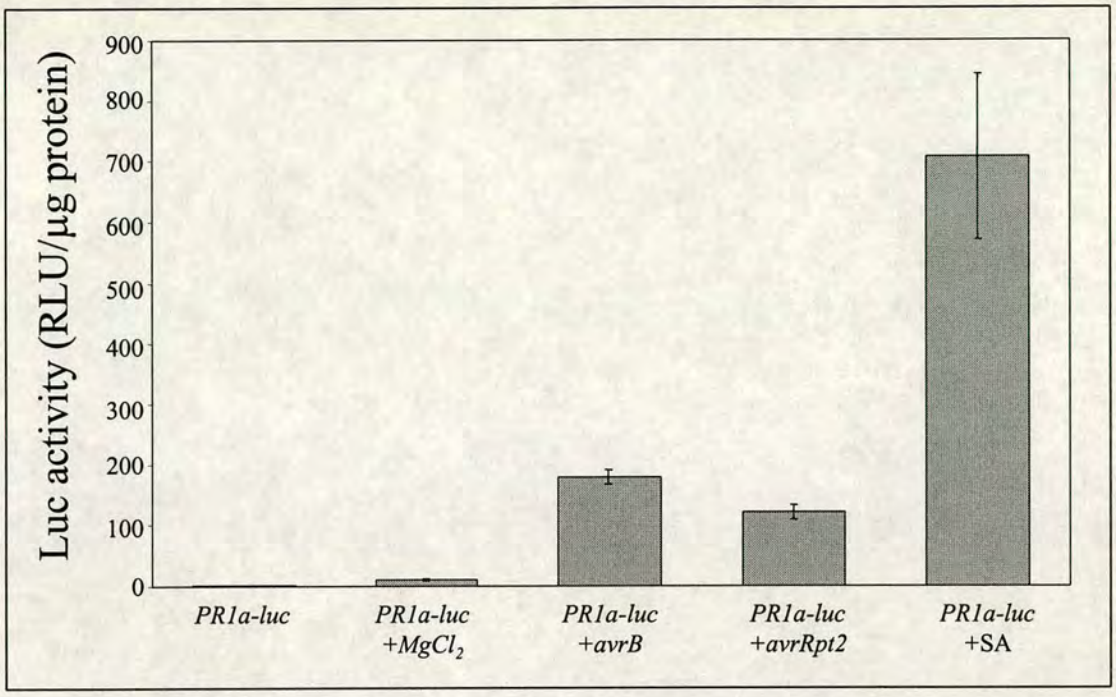

Fig.3.2. Luc activity in *PR-1a:luc* plants.
Plants were inoculated with 10mM $MgCl_2$, *Pst*DC3000 (*avrB*), *Pst* DC3000 (*avrRpt2*) or 1mM SA. Naive plants were included as a control. Leaves were harvested 72 hours after the treatment, or after 24 hours in the case of SA application. The values presented are an average of readings from four plants. Error bars represent the standard error between values at the 95% confidence level.

PR-1a:luc was expressed in transgenic Arabidopsis plants in response to inoculation with two avirulent bacterial pathogens or application of SA (Fig.3.2). The naïve control *PR-1a:luc* plants did not show an appreciable increase in luc activity (2 RLU/µg protein) and *PR-1a:luc* plants inoculated with $MgCl_2$ showed a very small increase in luc activity (10 RLU/µg protein) (Fig.3.2). Both *Pst*DC3000 *(avrB)* and *Pst*DC3000 *(avrRpt2)* induced approximately a 60-fold increase in luc activity in *PR-1a:luc* plants in comparison to naive *PR-1a:luc* plants, whereas SA application induced a 350-fold increase in luc activity (Fig.3.2). Thus, luc activity in *PR-1a:luc* plants is produced by conditions known to induce *PR-1* during the establishment of SAR. Luc activity can thus be taken as an accurate report of *PR-1* expression during the establishment of acquired resistance in local tissue.

The local spatial-temporal expression of *PR-1* in leaves inoculated with *Pst*DC3000 (*avrB*) was investigated in *PR-1a:luc* plants. Fig.3.3 is a time course showing the induction of *PR-1a:luc* expression in the 72 hours following inoculation with

*Pst*DC3000 (*avrB*). Luc activity began 12 hours after pathogen inoculation and appeared as a streak of light in the midrib of the leaf (Fig.3.3). By 24 hours post-inoculation, luc activity had spread to a 'C-shape' surrounding the HR, which is visible at this time point. The intensity of light produced in this area increased over time to 72 hours post-inoculation (Fig.3.3).

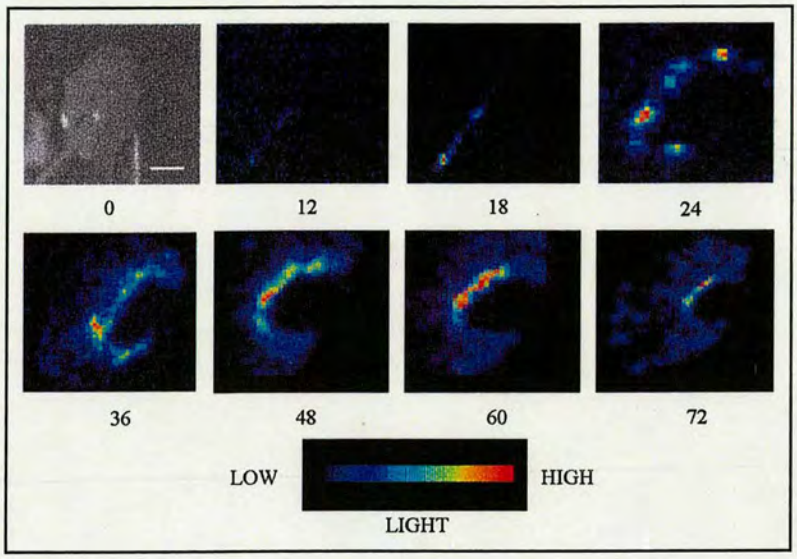

Fig.3.3. Camera images showing the induction of luc activity in *PR-1a:luc* leaves over time, following inoculation with *Pst* DC3000 *(avrB).*

Leaves were painted with 1mM luciferin and images were collected for 10s in the ultra low-light imaging camera. Inoculated leaves were imaged at the time points shown (in hours). Size bar represents 5 mm.

In order to quantify *PR-1a:luc* expression after inoculation with *Pst*DC3000 (*avrB*), a luminometer assay was performed. *PR-1a:luc* plants were inoculated with *Pst*DC3000 (*avrB*) and infected leaves were harvested every 12 hours for four days after inoculation. Protein extractions were performed and luc activity determined. Luc activity, calculated as RLU per microgram of extractable protein produced during the 20 seconds following luciferin injection, was plotted against time (Fig.3.4). *PR-1a:luc* expression peaked at 36 hours after inoculation with *Pst*DC3000 (*avrB*), which corresponded to the 'C-shape' of luc activity produced around the visible HR at this time (Fig.3.3). Luc activity was approximately 100-fold more than naïve *PR-1a:luc* plants at this time point (Fig.3.4). At 72 hours (3

days), luc activity was still induced to approximately 50-fold more than in naïve *PR-1a:luc* plants. By 96 hours post-inoculation, luc activity had decreased (Fig.3.4). A similar pattern of luc activity was visualised in Fig.3.3 and Fig.3.4, indicating that consumption of luciferase during the time course in Fig.3.3 did not limit luc activity. Northern blot analysis confirmed that luc activity produced in *PR-1a:luc* plants corresponded to the expression of *PR-1* in Col-0 following inoculation with *Pst* DC3000 *(avrB)* (Chini, unpublished results not shown).

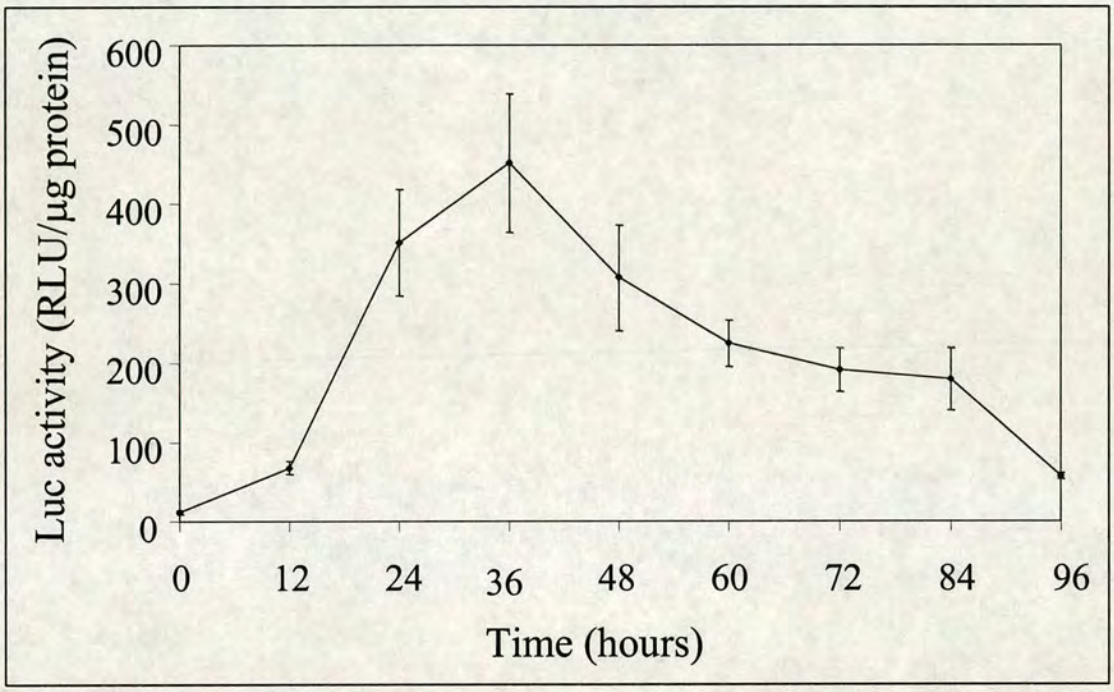

Fig.3.4. The extent of luc activity produced by *PR-1a:luc* plants over time, following inoculation with *Pst*DC3000 (*avrB*).

The values presented are an average of readings from four plants. Error bars represent the standard error between values at the 95% confidence level.

Attempts where made to visualise the expression of *PR-1a:luc* in systemic leaves following inoculation with *Pst*DC3000 *(avrB)*. Although it did appear that luc activity decreased in locally infected leaves after three days and light was observed in adjacent leaves, no clear pattern of *PR-1a:luc* expression was established (results not shown).

3.4. Mutagenesis of *PR-1a:luc* transgenic plants and isolation of candidate SAR mutants.

Production of mutants

Homozygous T2 seed (approximately 10 000) were subjected to ethylmethane sulfanate (EMS) mutagenesis (Redei & Koncz 1992) (Thomson & Loake, unpublished results). EMS-mutagenised seed were grown in 12 trays (8 pots per tray). Plants were allowed to self-fertilise (thus allowing all plants carrying recessive mutant genes to become homozygous) and the M2 seed was collected. Seed from each pot was pooled.

Primary screen

M2 seed was used for the mutant screen. In order to allow for ease of screening, the seed was divided into three groups:

Group A: Seed from tray 1,2,3 (plants screened November-December 1997)

Group B: Seed from tray 4,5,6 (plants screened January 1998)

Group C: Seed from tray 7,8,9,10,11,12 (plants screened February-March 1998).

Approximately 60 seeds from each pot were planted out. This amounted to 480 seeds per tray and a total of 5760 seeds.

The primary screen consisted of inoculating 4-5 week old plants, which had not yet started to bolt, with *Pst*DC3000 (*avrB*). After three days, plants were imaged using the ultra low-light imaging camera for abnormal luc activity. Two categories of candidate mutants were isolated: plants that did not produce luc activity and plants that produced increased luc activity in comparison to *PR-1a:luc* plants (Fig.3.3). For ease of reference, the first group of candidate mutants was called *neb* (*n*o *e*xpression of *b*ioluminescence) and the second group was called *heb* (*h*igh *e*xpression of *b*ioluminescence).

The 'mutant status' of the candidate mutant plants was verified by a second inoculation one to five days later. Candidate mutants were allowed to self-fertilise and the M3 seed was collected for further screening and confirmation of the mutant status in the next generation.

Secondary screen

The secondary screen was conducted on M3 plants from putative mutant lines. A total of 104 *heb* and 256 *neb* candidate mutants were re-tested in the secondary screen. The four steps comprising the secondary screen were:

1) A leaf from 3 different plants from each line was inoculated with *Pst*DC3000 (*avrB*) and imaged after three days. If all three leaves correlated to the result obtained in the primary screen, the line was tested further. If not, it was discarded.

2) The *neb* plants were tested for SA-induction of *PR-1a:luc* expression. SA (1 μM) was applied to the *neb* lines in order to determine their position relative to SA in the SAR pathway. Treatment of Arabidopsis with SA has previously been shown to induce *PR-1* expression after 24 hours (Uknes et al. 1992). A leaf from three different plants from each line was tested and imaged after 24 hours. Luc activity was determined and compared to SA-induction of luc activity in *PR-1a:luc* plants. Plants with luc activity corresponding to the wild type indicated that the probable mutation may be upstream of SA in these plants, where as plants that did not show luc activity after application of SA indicated that the block was likely downstream of SA.

3) One possibility that the *neb* plants did not show induction of luc activity following inoculation with *Pst*DC3000 (*avrB*) was that the *PR-1a:luc* transformation cassette (Fig.3.1) had been eliminated from the plants. In order to check that the *neb* plants still contained the transformation cassette, seeds from these plants were plated out on MS medium containing kanamycin. After 10 days, seedlings were scored for kanamycin resistance or susceptibility. Only plants showing 100% kanamycin resistance were selected as *neb* candidate mutants. Plants that did not show kanamycin resistance were discarded.

4) The *heb* plants were tested for constitutive luc activity. Three naïve *heb* plants from each line were painted with luciferin and imaged in the ultra low-light imaging camera. Plants that showed constitutive luc activity (i.e. induction of luc activity in

the absence of SAR induction) were placed in a new category, termed *ceb* (*c*onstitutive *e*xpression of *b*ioluminescence).

Table 3.1. Numbers of candidate mutant plants isolated in the mutant screen.

Categories	Number of plants
ceb	5
heb	3
neb - block upstream of SA	10
neb - block downstream of SA	19
Total	37

A total of 37 putative mutants were isolated by the end of the secondary screen (Table 3.1). A representative *ceb*, *heb* and *neb* candidate mutant is shown in Fig.3.5. Leaves were imaged for luc activity in the ultra low-light camera. The pattern of luc activity three days after inoculation with *Pst*DC3000 (*avrB*) is shown for the *neb* candidate (Fig.3.5b) and the *heb* candidate (Fig.3.5c) in comparison to *PR-1a:luc*, which displayed a 'C-shape' of light surrounding the HR (Fig.3.5a). No light was seen for the *neb* candidate whereas the band of light in the *heb* candidate was larger than in the *PR-1a:luc* plant (Fig.3.5). A naïve leaf from a *ceb* candidate plant showing constitutive luc activity is shown (Fig.3.5d).

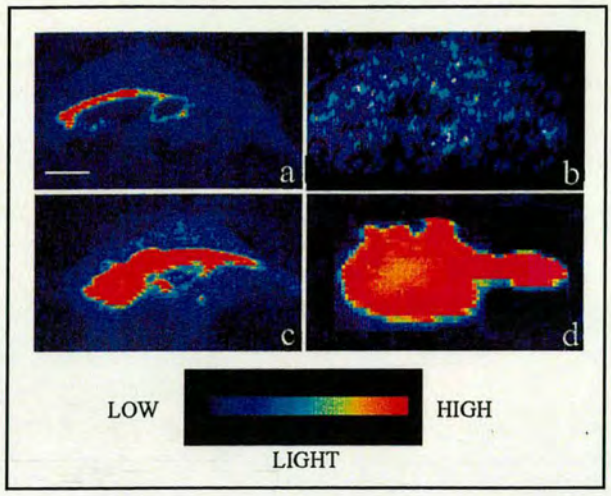

Fig.3.5. Examples of light production in individual leaves from the three classes of putative mutants in comparison to a *PR-1a:luc* plant.

a: *PR-1a:luc*, b: *neb1*, c: *heb1* and d: *ceb1*. Leaves in a,b and c were inoculated with *Pst* DC3000 *(avrB)* and imaged after three days. All leaves were painted with 1mM luciferin and images were collected for 10s in the ultra low-light imaging camera. Size bar represents 5 mm.

3.5. Discussion

A transformation cassette containing the –903 bp *PR-1a* promoter from tobacco (Payne et al. 1988) was fused to the *luc* reporter gene and the cassette was used to transform Arabidopsis. Luc activity was induced in a homozygous transgenic line by SA and inoculation with avirulent bacterial pathogens (Fig.3.2). As SAR induction by SA and inoculation with an avirulent pathogen has been well described (Ryals et al. 1996), luc activity in *PR-1a:luc* plants can thus be taken as a report of the establishment of SAR. Expression of the *PR-1a* promoter has previously been studied in transgenic tobacco. The –903bp *PR-1a* promoter was fused to the β-glucoronidase (GUS) reporter protein encoded by the *uidA* gene and used to transform tobacco (Van de Rhee et al. 1990, Uknes et al. 1993). In both cases inoculation of the transgenic plants with TMV or application of SA induced expression of *PR-1a:uidA* (Van de Rhee et al. 1990, Uknes et al. 1993). Thus, expression of the *luc* reporter gene in the *PR-1a:luc* transgenic Arabidopsis line corresponded to GUS expression in *PR-1a:uidA* transgenic tobacco lines, viz. that expression of the reporter gene was induced by both SA and inoculation with an avirulent pathogen.

Following inoculation of *PR-1a:luc* plants with *Pst*DC3000 (*avrB*), luc activity was produced around the HR (Fig.3.3). This result corresponded to TMV induction of GUS activity in *PR-1a:uidA* transgenic tobacco, where GUS activty was greatest in the area adjacent to the TMV lesions (Uknes et al. 1993). GUS activity was also observed surrounding the HR produced when *PR-1a:uidA* transgenic tobacco leaves were inoculated with a 50mM glycoprotein extract from *Phytophthora megasperma* (Costet et al. 1999). Thus, expression of a reporter gene under the control of the *PR-1a* promoter in plants is associated with cell death following the interaction of *R-avr* gene products, an increase in available SA and the establishment of acquired resistance. Inoculation of *PR-1a:luc* Arabidopsis plants with $MgCl_2$ did not induce luc activity (Fig.3.2), nor did rubbing of *PR-1a:uidA* tobacco plants with carborundum induce GUS activity (Uknes et al. 1993).

Luc activity following inoculation of *PR-1a:luc* plants with *Pst*DC3000 (*avrB*) was detected after 12 hours (Fig.3.3 & Fig.3.4). Luc activity increased sharply after 24 hours, peaking at 36 hours post-inoculation, and decreased after 96 hours (Fig.3.4). The rapid induction of luc activity 12 to 24 hours post-inoculation is consistent with *PR-1* expression in Arabidopsis following inoculation with the avirulent bacterial pathogen *P. syringae* pv *maculicola* ES4326 (*avrRpt2*) (Reuber & Ausabel 1996). Expression of the *PR-1* gene in Arabidopsis Col-0 also increased 12 to 24 hours post-inoculation with *Pst*DC3000 (*avrB*), peaked at 36 hours and decreased after 96 hours (Chini, unpublished results), further indicating that luc activity accurately reported *PR-1* expression during the establishment of SAR.

Recently, a -4258 bp Arabidopsis *PR-1* promoter was isolated and the *cis*-acting regulatory elements involved in INA induction were characterised by deletion analysis, linker-scanning mutagenesis and *in vivo* footprinting (Lebel et al. 1998). It was found that a -621bp promoter was essential for INA-inducible *PR-1* expression. Linker-scanning analysis showed that this region of the promoter contained a CGTCA motif, which has high homology to the recognition site of transcription factors of the basic leucine zipper class (Lebel et al. 1998). As INA is an analogue of

SA, it is likely that elements in the *PR-1* promoter that are necessary for INA-inducibility, are also required for SA-induction of *PR-1* expression. Deletion analysis of the *PR-1a* promoter indicated that a -661 bp minimal region (Uknes et al. 1993) or a -643 bp minimal region (Van de Rhee et al. 1990) was essential for SA-induction of the GUS reporter gene. In addition, functional analysis of the *PR-1a* promoter revealed a region of 139 bp (from –691 to –553) required for GUS expression in response to SA (Strompen et al. 1998). This region contained two TGACG motifs (the complementary sequence of CGTCA) which were found to be the recognition site of TGA1a, a tobacco transcription factor of the basic leucine zipper class (Strompen et al. 1998). This indicates that a similar core region of both the *PR-1* and *PR-1a* promoters are required for SA-inducibility. Furthermore, both genes appear to be under the control of a similar *cis*-acting regulatory element.

Homozygous *PR-1a:luc* seed was mutagenised and M2 candidate mutant plants showing perturbations in the pattern of luc activity following inoculation with *Pst*DC3000 *(avrB)* were identified. A total of 37 candidate mutants were isolated following secondary screening of M3 plants (Table 3.1). The candidate mutants could be divided into various classes (Fig.3.5). Characterisation of a group of these mutants will be presented in Chapter 4.

Chapter Four

Characterisation of *neb* and *ceb* candidate mutants

4.1. Introduction

Two groups of candidate mutants identified in this study and thought to represent new SAR mutant classes were chosen for further characterisation. The *neb* candidate mutants with a putative block upstream of SA in the SAR signal transduction pathway and the *ceb* candidates were studied further. A number of mutants have previously been uncovered that show constitutive expression of *PR-1*. However, for many of these mutants such as *lsd1* (Dietrich et al. 1994), *acd2* (Greenberg et al. 1994), *cpr5* (Bowling et al. 1997) and *cep* (Silva et al. 1999), constitutive *PR-1* gene expression is associated with the spontaneous formation of HR-like lesions. The *ceb* candidate mutants uncovered in this project did not display spontaneous formation of visible lesions, or accelerated cell death following inoculation with *Pst*DC3000 *(avrB)*, and thus represent a mutant class where cell death and constitutive *PR-1* expression have been uncoupled. Furthermore, a number of different alleles of *NPR1/NIM1,* which acts downstream of SA in SAR, have already been uncovered in mutant screens (Cao et al. 1994, Delaney et al. 1995, Glazebrook et al. 1995, Shah et al. 1997). In order to avoid isolating further alleles of *NPR1*, it was decided to characterise the group of *neb* candidate mutants that appeared to be upstream of SA in the SAR signal transduction network.

The *ceb* and *neb* candidate mutants were studied using three approaches in order to determine if they were true SAR mutants. Firstly, the extent of *PR-1a:luc* expression in the mutants was quantified using the luminometer method. Secondly, the expression of SA-dependent defence genes (*PR-1, PR-2* and *PR-5*) (Uknes et al. 1992), jasmonate-dependent genes (*Thi2.1, LOX2*) (Bohlman et al. 1998, Bell & Mullet 1993), a gene dependent on concominant signalling through jasmonates and ethylene (*PDF1.2*) (Penninckx et al. 1998) and *ACS2*, a gene encoding a biosynthetic intermediate in the production of ethylene (Liang et al. 1992), were determined using Northern blot analysis. Thirdly, the resistance of the candidate mutants to a range of

plant pathogens normally virulent on the Col-0 ecotype was determined. Disease resistance assays with the bacterial pathogen *Pseudomonas syringae* pv. *tomato* (Whalen et al. 1991), the biotrophic oomycete pathogen *Peronospora parasitica* Noco2 (Parker et al. 1993) and the necrotrophic fungal pathogen *Fusarium oxysporum* f.sp. *matthiolae* (Mauch-Mani & Slusarenko 1994) were performed.

4.2. **Luminometer assays**

neb candidate mutants

Four *neb* candidate mutants were selected for initial further study. They were *neb26, neb27, neb28* and *neb29.* Fig.4.1 represents the results from luminometer assays of these four mutants and the *PR-1a:luc* transgenic line under various conditions: 36 hours after inoculation with *Pst*DC3000 *(avrB)* (a), 72 hours after *Pst*DC3000 (*avrB*) inoculation (b) and 24 hours after 1mM SA application (c). The 36 hours post-inoculation time point was chosen as *PR-1a:luc* expression was previously found to peak at this time following inoculation with *Pst*DC3000 *(avrB)* (Fig.3.3). The 72 hours post-inoculation time point was chosen in order to determine if the *neb* candidate mutants showed delayed increase in luc activity following inoculation with *PstDC3000 (avrB).*

The four *neb* candidate mutants all showed a dramatic reduction in luc activity at both 36 and 72 hours post-inoculation with *Pst*DC3000 *(avrB)* in comparison to *PR-1a:luc* plants (Fig.4.1a&b). The four *neb* candidates expressed *PR-1a:luc* 24 hours after application of 1mM SA (Fig.4.1c). However, the amount of luc activity in the *neb* candidates was approximately two to seven times less than the extent of luc activity produced in the *PR-1a:luc* wild-type plants treated with SA (Fig.4.1c). In comparison, GUS activity in SA treated *npr1* mutant plants (which contain the *PR-2* promoter fused to the *uidA* gene) was approximately 15 times less than SA treated wildtype transgenic plants (Cao et al. 1994).

The luminometer results presented in Fig.4.1 suggest that these *neb* candidate mutants are blocked in *PstDC3000 (avrB)* induction of luc activity, but not in SA

induction of *PR-1a:luc* expression, and thus may define mutations in the SAR signal transduction pathway upstream of SA. Furthermore, the luminometer results presented in Fig.4.1 confirm the results obtained using the ultra low-light imaging camera to image luc activity in *neb* plants, which was carried out during the secondary phase of the mutant screen.

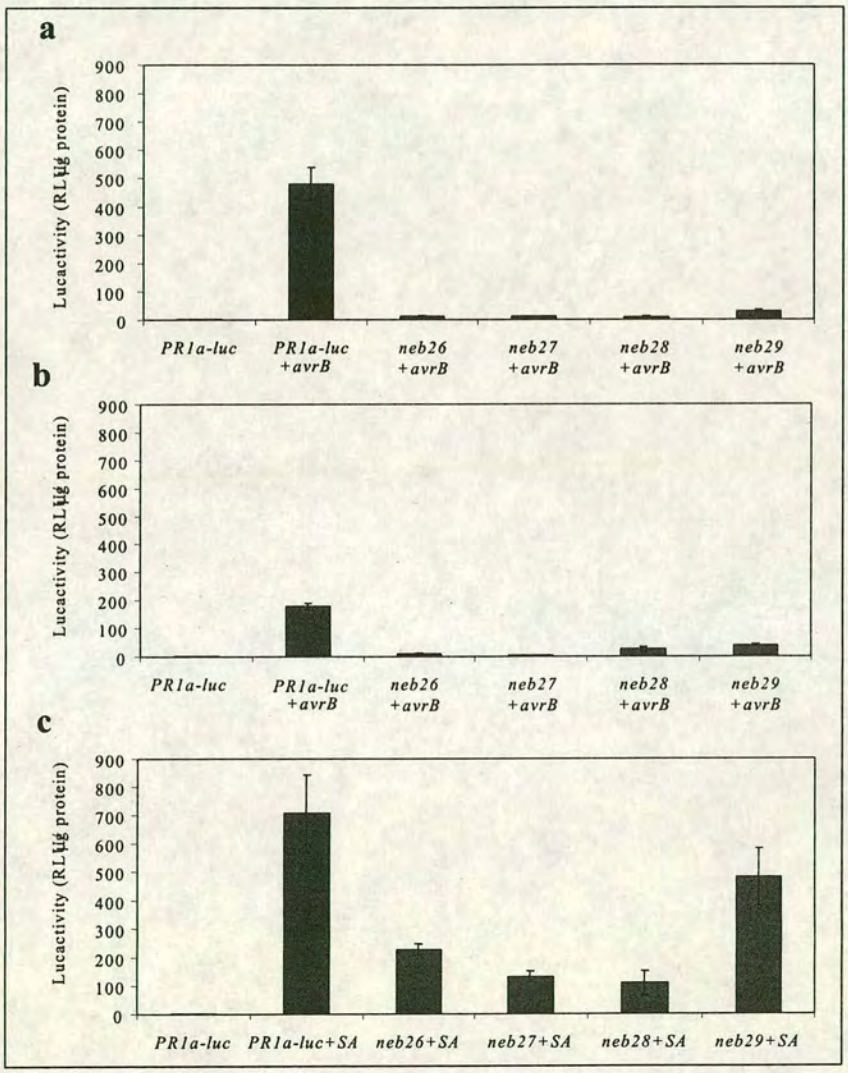

Fig.4.1. Luminometer assay of *neb* candidate mutants, assayed either 36 hours after inoculation with *Pst*DC3000 *(avrB)* (a) or after 72 hours (b), and 24 hours after application of 1mM SA (c).

Each graph includes untreated control *PR-1a:luc* plants. The values presented are an average of readings from four plants. Error bars represent the standard error between values at the 95% confidence level. Luminometer assays were repeated twice and results obtained followed a similar trend.

ceb candidate mutants

All five *ceb* candidate mutants were selected for further study. They were *ceb1, ceb3, ceb4, ceb5* and *ceb6.* All five candidate mutant lines showed no morphological changes, except for *ceb5* which displayed earlier senescence in comparison to wild-type Col-0 plants (results not shown). None of the plants showed macroscopic HR lesion production. Fig.4.2 represents the results from luminometer assays of naïve five-week old plants of all five candidates and the *PR-1a:luc* transgenic line. *PR-1a:luc* plants sprayed with 1mM SA 24 hours prior to the luminometer assay were also included (Fig.4.2). As expected, *PR-1a:luc* plants treated with SA showed an increase in luc activity in comparison to naïve *PR-1a:luc* plants (Fig.4.2).

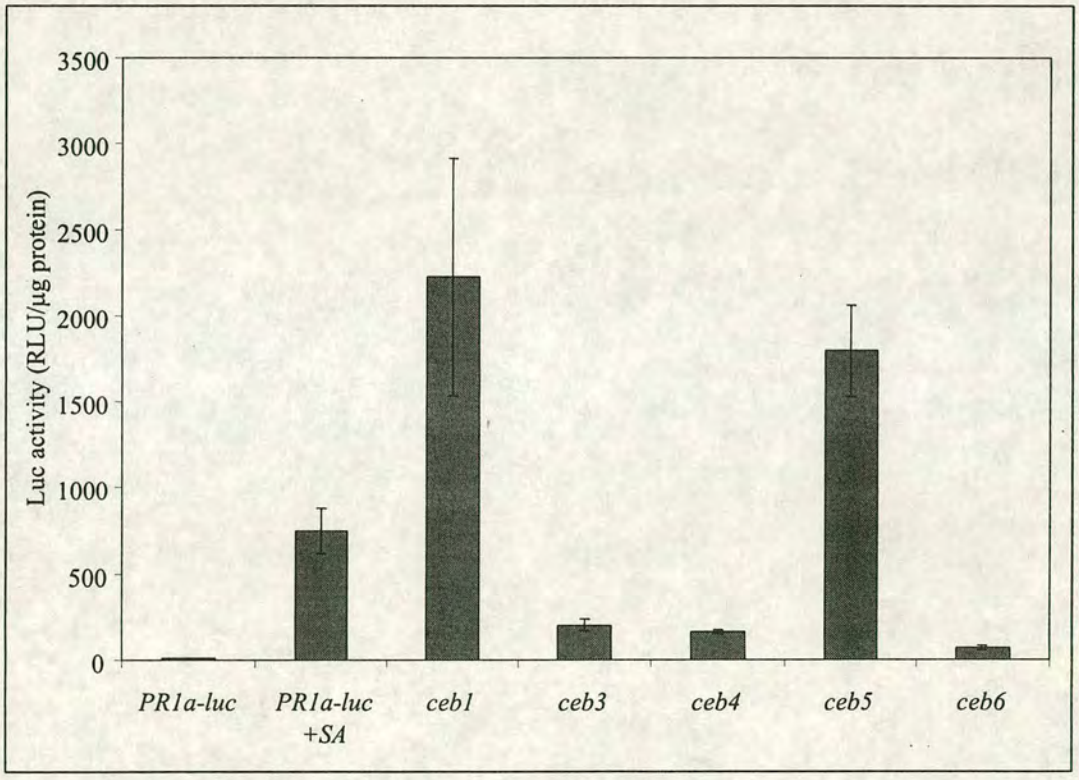

Fig.4.2. Luminometer assay of *ceb* candidate mutants

Naïve five-week old plants were assayed, and *PR-1a:luc* leaves treated with 1mM SA 24 hours prior to harvesting were included as a control. The values presented are an average of readings from four plants. Error bars represent the standard error between values at the 95% confidence level. The luminometer assay was repeated twice and results obtained followed a similar trend.

All five *ceb* candidate mutants showed constitutive expression of *PR-1a:luc* in comparison to *PR-1a:luc* plants, ranging from five-fold greater expression in the case

of *ceb6* to more than 100-fold in the case of *ceb1* and *ceb5* (Fig.4.2). Only *ceb1* and *ceb5* expressed *PR-1a:luc* to levels higher than the SA-treated *PR-1a:luc* leaves (Fig.4.2). The *cpr1* and *cpr5* mutants, which express constitutive SAR and also contain the *PR-2* promoter fused to the *uidA* gene, showed a two-fold increase in GUS activity in comparison to naïve transgenic plants (Bowling et al. 1994, Bowling et al. 1997). Although it is difficult to make a direct comparison between the *ceb* and *cpr* mutants as different SAR promoter- marker transcriptional fusions were used, *ceb1* and *ceb5* do show a greater increase in marker protein expression in comparison to published reports of other SAR mutants.

4.3. **Northern blot analysis**

neb candidate mutants

Expression of the endogenous Arabidopsis *PR-1* gene (Uknes et al. 1992) was determined in the four *neb* candidate mutants. This analysis was performed in order to confirm that the loss of luc activity following *Pst* DC3000 *(avrB)* inoculation accurately depicted *PR-1* gene expression. Col-0 wildtype plants, *nahG*, *npr1* and the *neb* candidates were inoculated with *Pst*DC3000 (*avrB*). Inoculated leaves were harvested after 48 hours and total RNA was extracted. RNA samples were separated by electrophoresis, transferred to a membrane by Northern blotting and the membrane was probed with *PR-1* and the ribosomal *18S* gene. The latter constitutive probe was included in order to ensure that uniform loading and transfer of RNA had taken place. The result is shown in Fig.4.3. As was expected, the uninfected Col-0 sample did not show *PR-1* activity, whereas Col-0 plants inoculated with *Pst*DC3000 *(avrB)* did (Fig.4.3). *nahG* plants, which accumulate reduced levels of SA following *Pst*DC3000 *(avrB)* infection (Delaney et al. 1994), did not show *PR-1* expression (Fig.4.3). The *npr1* mutant, which is blocked in SA induction of *PR-1* expression (Cao et al. 1994), showed a small amount of *PR-1* expression (Fig.4.3). The *jar1* plants inoculated with *Pst*DC3000 (*avrB)* expressed *PR-1* to levels comparable to wild-type Col-0. The *jar1* mutants are insensitive to jasmonate signalling (Staswick et al. 1992). Application of Me-JA did not induce *PR-1* expression (Fig.4.3). These results confirm previous observations that Me-JA plays no direct role in the signal

transduction pathway leading to *PR-1* expression (Penninckx et al. 1996). From the luminometer data, it was expected that the *neb* lines would show reduced *PR-1* expression following *Pst* DC3000 (*avrB*) inoculation. However, all four *neb* lines showed increased *PR-1* expression following *Pst*DC3000 *(avrB)* in comparison to *nahG* or *npr1* plants and naive Col-0 plants (Fig.4.3). All four *neb* lines also showed increased *PR-1* expression following SA application (results not shown). From these results it appears that the *neb* lines do not contain a loss-of- function mutation in the signal transduction pathway leading to the expression of the Arabidopsis *PR-1* gene.

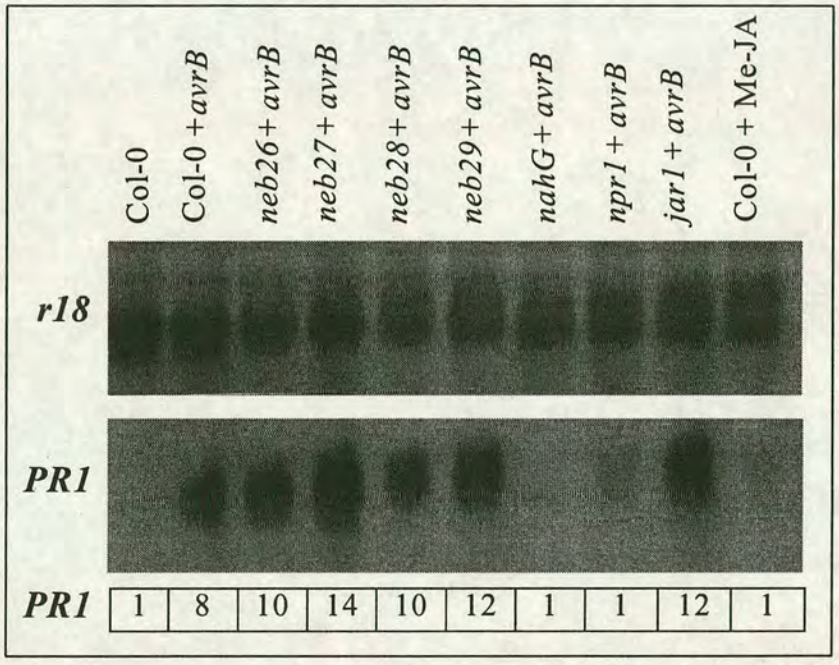

Fig.4.3. Northern blot analysis of *PR-1* mRNA expression in *neb* plants.

Col-0, *neb26, neb27, neb28, neb29, npr1, nahG* and *jar1* plants were inoculated with *Pst*DC3000 *(avrB)*. Wild-type plants (Col-0) were included as a negative control and Col-0 plants were treated with 100μM Me-JA. RNA samples were consecutively probed with the *PR-1* and *18S* probes. The table below the blot represents the fold induction of the gene expression for each sample relative to that of Col-0. The expression has been quantified using the ImageQuant software, adjusted to the expression of the loading control (*r18*) and normalised to the expression of Col-0, which was set to 1 unit. Northern blot analysis was repeated twice with similar results. *avrB,* plants inoculated with *Pst*DC3000 *(avrB)*.

ceb candidate mutants

Leaves from naïve five-week old Col-0 plants and all five *ceb* plants were harvested for Northern blot analysis. Col-0, *nahG, npr1* and *jar1* plants were inoculated with *Pst*DC3000 *(avrB)* and leaves were harvested after 48 hours. Col-0 plants were

treated with 1mM SA or 100μM Me-JA and leaves were harvested after 24 hours. Col-0 plants were also treated with 2mM H_2O_2 and leaves were harvested after 4.5 hours. Total RNA was extracted from all the samples and Northern blot analysis was performed. Expression of the SA-dependent genes *PR-1*, *PR-2* and *PR-5* (Uknes et al. 1992) and the SA-independent genes *PDF1.2* (Penninckx et al. 1996) and *Thi2.1* (Epel et al. 1995) were determined (Fig.4.4). Northern blot analysis was repeated at least twice on separate occasions and similar results were obtained. Expression of *PDF1.2* is dependent on jasmonate and ethylene signalling (Penninckx et al. 1998) and *Thi2.1* expression is dependent on jasmonate signalling (Bohlmann et al. 1998). *AtLOX2* and *ACS2* were also selected for Northern blot analysis (Fig.4.4). *AtLOX2* encodes a lipoxogenase enzyme which catalyses the hydroperoxidation of unsaturated fatty acids and is thought to be important in the biosynthesis of jasmonic acid (Bell & Mullet 1993). *AtLOX2* expression is rapidly induced in leaves following Me-JA application (Bell & Mullet 1993). Thus, a high level of expression of *AtLOX2* can be taken as an indication of the presence of accumulated jasmonates. *ACS2* encodes an isoform of ACC synthase and is the major form of the enzyme found in Arabidopsis vegetative tissues (Liang et al. 1992). ACC synthase is the rate-limiting enzyme responsible for conversion of methonine to ACC during the biosynthesis of ethylene (Kende 1993). Thus, increased transcription of *ACS2* may indicate an increase in ethylene production in Arabidopsis plants. The blots were also probed with the glutathione S-transferase 1(*GST1*) probe. GSTs are enzymes that catalyze the conjugation of glutathione to a variety of toxic substrates and in plants, a subclass of GSTs has been implicated in numerous stress responses, including oxidative stress and pathogen attack (Marrs et al. 1996). *GST1* expression is upregulated following inoculation with *Pst*DC3000 *(avrB)* and application of hydrogen peroxide (Grant & Loake 2000, in press).

All five *ceb* candidate mutants displayed constitutive expression of *PR-1, PR-2, PR-5, PDF1.2* and *GST1* but not *Thi2.1* (Fig.4.4a,b,c). All five candidates did not show substantially enhanced expression of *ACS2* and only *ceb5* showed enhanced expression of *AtLOX2 (*Fig.4.4c,d). As in Fig.4.3, control Col-0 leaves did not express *PR-1* whereas Col-0 and *jar1* leaves inoculated with *Pst*DC3000 *(avrB)* did

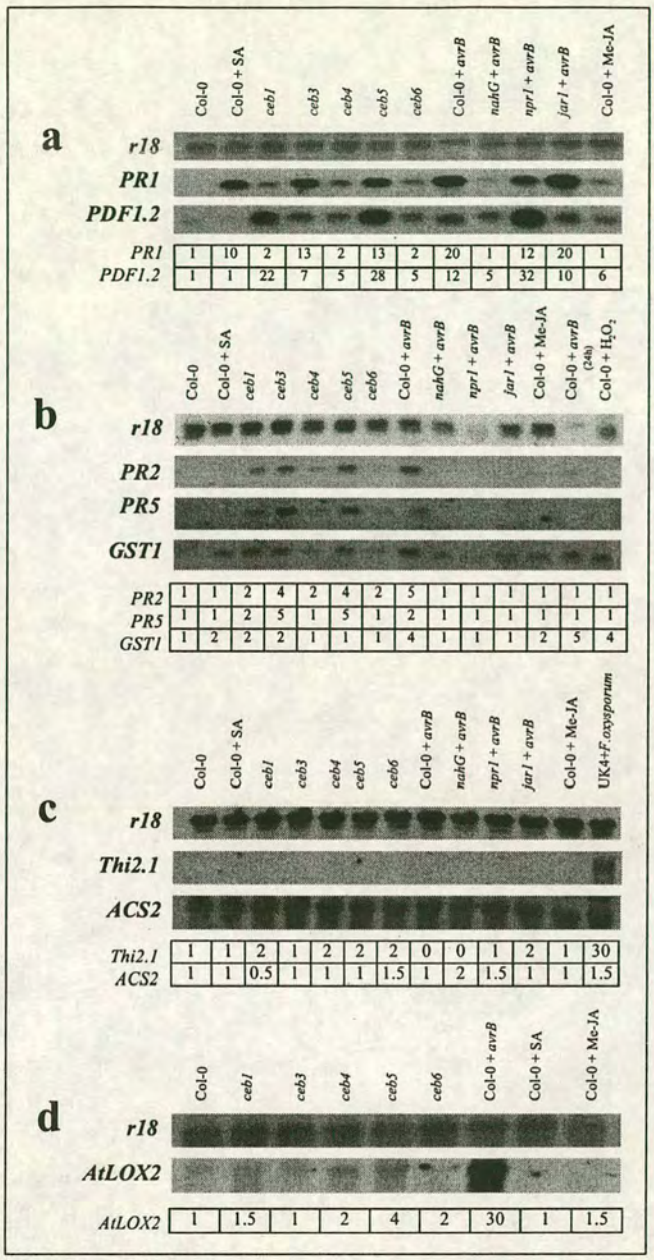

Fig.4.4. Northern blot analysis of defence mRNA expression in *ceb* plants: *PR-1, PDF1.2* (a), *PR-2, PR-5, GST1* (b), *Thi2.1* and *ACS2,* (c) and *AtLOX2* (d).

Naïve Col-0, *ceb1, ceb3, ceb4, ceb5* and *ceb6* leaves were harvested. As controls, Col-0, *nahG, npr1* and *jar1* plants were inoculated with *Pst*DC3000 *(avrB)* and leaves were harvested 48-hours post-inoculation, or Col-0 was treated with 1mM SA, 100μM Me-JA or 2mM H_2O_2. RNA samples were consecutively probed with the *PR-1, PDF1.2* and *r18* probes (a), the *PR-2, PR-5, GST1* and *r18* probes (b), the *Thi2.1, ACS2* and *r18* probes (c) and the *AtLOX2* and *r18* probes (d). The table below the blot represents the fold induction of the gene expression for each sample relative to that of Col-0. The expression has been quantified using the ImageQuant software, adjusted to the expression of the loading control (*r18*) and normalised to the expression of Col-0, which was set to 1 unit. Northern blot analysis was repeated twice in all cases with similar results. *avrB,* plants inoculated with *Pst*DC3000 *(avrB).*

(Fig.4.4a). Expression of *PR-1* in the *nahG* + *avrB* sample was negligible but was increased for the *npr1* + *avrB* sample (Fig.4.4a) in comparison to the first blot (Fig.4.3). The Col-0 control did not show expression of *PDF1.2, PR-2, PR-5, GST1* and *Thi2.1* as expected, and showed basal expression of *ACS2* and *AtLOX2* (Fig.4.4a,b,c,d). The Col-0+SA sample showed induced expression of *PR-1* and weak expression of *PR-2, PR-5* and *GST1* (Fig.4.4a&b), whereas the Col-0+*avrB* sample showed induced expression of *PR-1, PR-2, PR-5, GST1, PDF1.2* and *AtLOX2* but not *Thi2.1* (Fig.4.4a,b,c). From previous reports, it was expected that SA application would not induce *PDF1.2* expression (Penninckx et al. 1996). The Col-0+Me-JA sample showed weak induction of *PR-1, PDF1.2* and *GST1* expression (Fig.4.4a&b). Hydrogen peroxide application induced expression of *GST1* only (Fig4.4b). The induction of *GST1* expression by SA and Me-JA application most likely indicates a role for GST1 in the detoxification of these compounds. *GST1* expression was higher 24 hours after inoculation of Col-0 with *PstDC3000 (avrB)* than 48 hours post-inoculation, confirming previous observations that *GST1* is rapidly expressed following inoculation with an avirulent pathogen (Grant & Loake 2000, in press). *NahG* plants inoculated with *PstDC3000 (avrB)* showed slight induction of *PDF1.2* (Fig.4.4a). It has previously been found that *nahG* plants inoculated with *Alternaria brassisicola* expressed higher levels of *PDF1.2* than control *nahG* plants and this is thought to demonstrate a degree of cross-talk between the SA-dependent and SA-independent signal transduction pathways (Penninckx et al. 1996). In comparison to Col-0+*avrB*, the *npr1+avrB* sample showed increased expression of *PDF1.2* but decreased expression of *PR-1* (Fig.4.4a). The *jar1+avrB* sample showed a high level of *PR-1* expression but no increase in *PDF1.2* expression in comparison to Col-0+*avrB* (Fig.4.4a). This indicates that cross-talk between SA-dependent gene induction and Me-JA- dependent gene induction may be occurring in these two mutant lines. In Fig.4.4c a positive control for *Thi2.1* expression was included. Plants of Arabidopsis ecotype UK4 were inoculated with *Fusarium oxysporum* f.sp. *matthiolae* and leaves for RNA extraction were harvested after five days. Previously it has been shown that *Thi2.1* is expressed following *F.oxysporum* f.sp. *matthiolae* infection in UK4 leaves but not in Col-0 (Epple et al.

1998). Here the UK4 + *F.oxysporum* f.sp. *matthiolae* sample was the only sample to express *Thi2.1* (Fig.4.4c).

4.4. Disease resistance assays

Both the *neb* and *ceb* candidate mutant lines were tested for resistance to the bacterial pathogen *Pseudomonas syringae* pv *tomato* DC3000 (*Pst*DC3000) and the virulent oomycete pathogen *Peronospora parasitica* Noco2. *Pst*DC3000 is the causal organism of bacterial speck of tomato and bacterial growth is usually limited to the locally infected leaf (Agrios 1997). *Pst*DC3000 is virulent on the Arabidopsis ecotype Col-0 (Whalen et al. 1991) and induced resistance to this bacterial pathogen is associated with SA accumulation and *PR-1* expression (Cameron et al. 1999). *Peronospora parasitica*, a member of the oomycete family *Peronosporaceae*, is an obligate biotroph and causes downy mildew in *Cruciferae* (Agrios 1997). Many *P.parasitica* isolates have been uncovered with varying degrees of resistance to Arabidopsis ecotypes (Holub et al. 1994). *P.parasitica* Noco2 is virulent on the ecotype Col-0 (Parker et al. 1993) and thus was chosen for the disease resistance analysis. Induced resistance to *P.parasitica* Noco2 in Col-0 plants is also associated with SA accumulation and *PR-1* expression (Bowling et al. 1994, Bowling et al. 1997, Clarke et al. 1998). It has also been suggested that *PDF1.2* expression may play a role in *P.parasitica* induced resistance in Col-0 (Bowling et al. 1997). As the *neb* candidate mutants showed loss of *PR-1a:luc* expression, it was expected that they would show enhanced susceptibility to these two pathogens. On the other hand, the *ceb* candidate mutants showed constitutive expression of *PR-1* and *PDF1.2* and thus were expected to show enhanced resistance. The *ceb* candidates only were tested for resistance to the necrotrophic fungal pathogen *Fusarium oxysporum* f.sp.*matthiolae* (Mauch-Mani & Slusurenko 1994).

Pseudomonas syringae pv *tomato* DC3000.

Five-week old Col-0, *nahG*, *npr1*, *ceb* and *neb* plants were inoculated with *Pst* DC3000. Development of disease symptoms (chlorosis, wilting of inoculated leaves) was monitored daily and development of symptoms in wildtype Col-0 was found to

be optimal at four days post-inoculation (Fig.4.5a). At this time period *nahG* plants had undergone extreme chlorosis and wilting in comparison to Col-0. The *neb* and *ceb* candidate mutants showed varying degrees of chlorosis (results not shown), and so it was decided to quantify bacterial growth in these mutant lines in order to compare them to Col-0. Three plants per line were inoculated with *Pst*DC3000 and leaves were harvested after three days. This time point was chosen as *Pst*DC3000 growth in Arabidopsis peaks three days after inoculation (Whalen et al. 1991, Delaney et al. 1994). Only *neb26, neb27* and *neb29* were tested from the *neb* lines, as insufficient seed was collected for *neb28*. Leaf disks were produced from each inoculated leaf, ground in 10mM $MgCl_2$, dilutions were made and plated out on KB media. After three days, the number of bacterial colonies were recorded as an indication of the bacterial titre in the different plants. Fig.4.5b&c outlines the results obtained for both the *ceb* and *neb* candidate mutants respectively.

In Fig.4.5b, the Col-0 samples showed a bacterial titre of 10^7 cfu/leaf disk which corresponds to bacterial titres in Col-0 at this time point in previous studies (Bowling et al. 1997, Clarke et al. 1998). Bacterial titres in *nahG* and *npr1* plants were greater than Col-0, which also corresponds to previous reports (Bowling et al. 1997). In both the case of *nahG* and *npr1*, a statistically significant difference in bacterial titre in comparison to Col-0 was found using the Mann-Whitney test at the 95% confidence level (Mini-tab Version 12). The *neb26, neb27* and *neb28* candidate mutant lines showed bacterial titres more similar to Col-0 than *npr1* or *nahG* and thus do not appear to show an 'enhanced disease susceptibility' phenotype to *Pst*DC3000 (Fig.4.5b).

The bacterial titre in the Col-0 samples in the *ceb* experiment (10^5 cfu/leaf disk, Fig.4.5c) was less than the bacterial titre in the *neb* experiment (10^7 cfu/leaf disk, Fig.4.5a). It has previously been shown that bacterial titres in Col-0 plants can vary significantly in different experiments (Glazebrook et al. 1996). However, the same trend of *Pst*DC3000 growth can be seen in both Fig.4.5b and Fig.4.5c. In both

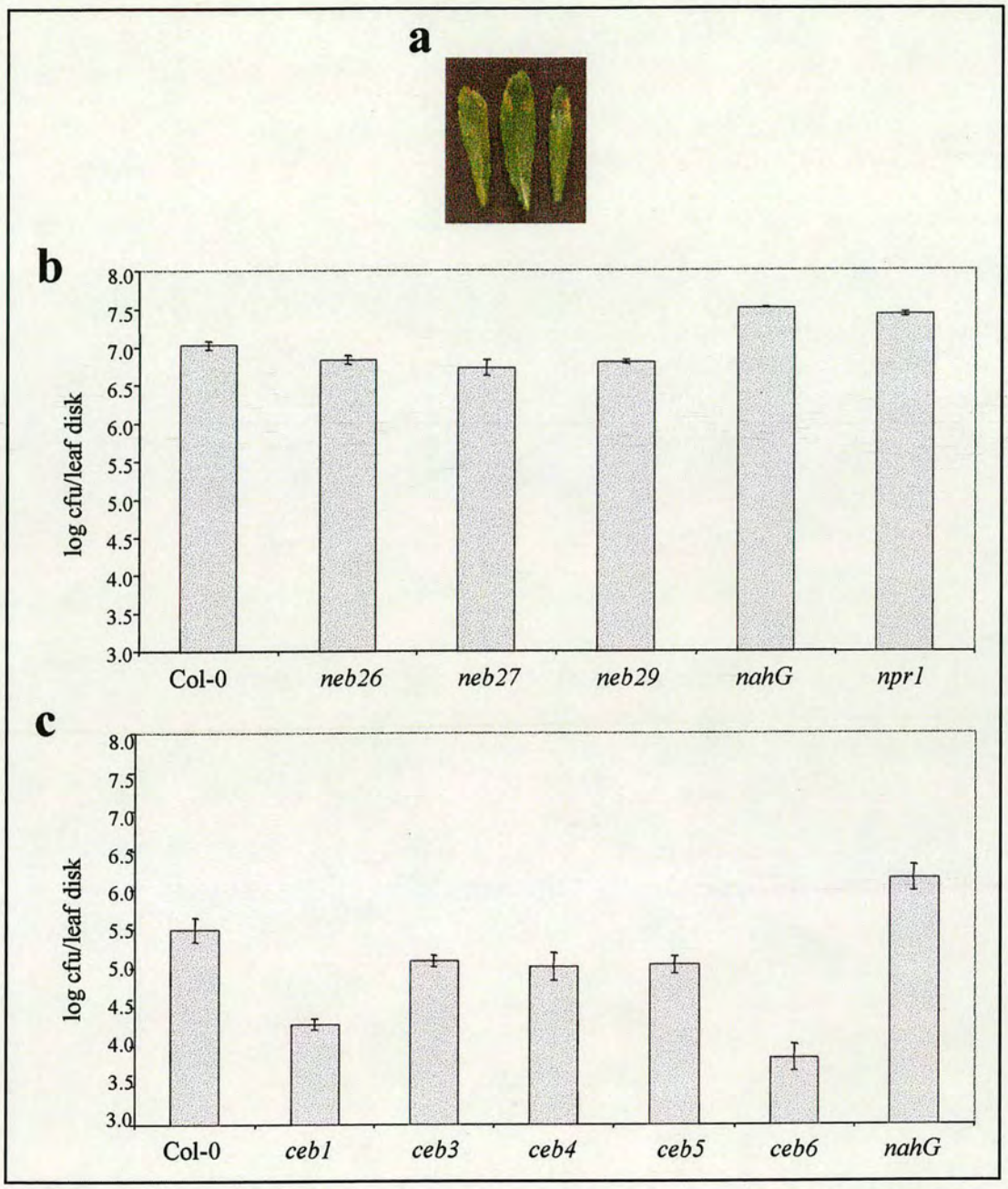

Fig.4.5. Resistance of *neb* and *ceb* candidate mutants to *Pst*DC3000.

a: The development of *Pst*DC3000 symptoms on Col-0 4 days after inoculation.
b: Graph indicating bacterial titre three days after *Pst*DC3000 inoculation. Col-0, *nahG*, *npr1* and three *neb* candidate mutants were tested.
c: Graph indicating bacterial titre three days after *Pst*DC3000 inoculation. Col-0, *nahG* and all five *ceb* candidate mutants were tested.
In all cases, values presented are the averages obtained from three plants and error bars represent the standard error between values at the 95% confidence level. Experiments were repeated twice and results obtained followed a similar trend.

experiments, the bacterial titre in *nahG* plants was greater than in Col-0 plants. The *ceb3, ceb4* and *ceb5* lines showed bacterial titres equivalent to those of Col-0 (Fig.4.5c) and *ceb1* and *ceb6* showed bacterial titres significantly less than Col-0 (Fig.4.5c). The reduction in bacterial titre in *ceb1* and *ceb6* corresponds to results for *cpr5* and *cpr6*, where bacterial titres were also reduced in the mutant lines by 1000 cfu per leaf disk in comparison to Col-0 (Bowling et al. 1997, Clarke et al. 1998).

Peronospora parasitica Noco2

Four-week old Col-0, *nahG, npr1, ceb* and *neb* plants were spray-inoculated with a *P.parasitica* Noco2 conidiospore suspension. The plants were maintained in a humid environment for 10 days before they were visually accessed for the extent of *P.parasitica* Noco2 growth. Plants were grown in three separate trays, with four plants in each tray. After 10 days, Col-0 plants typically showed downy mildew symptoms (Fig.4.5a, visualisation of sporulating conidiophores). The extent of macroscopic condiophore development over the surface area of the leaves was scored under good light (scoring criteria outlined in Chapter 2). A disease index was applied to the scores and the results obtained are outlined in Fig.4.6b&c.

In both Fig4.6b and 4.6c, *nahG* and *npr1* plants showed a greater degree of conidiophore development in comparison to Col-0. This confirms results previously obtained for *npr1* and Col-0 (Bowling et al. 1997). None of the *neb* lines showed an increase in conidiophore development in comparison to Col-0 (Fig.4.6b). The *ceb1, ceb3, ceb4* and *ceb6* lines showed a reduction in conidiophore development in comparison to Col-0, whereas *ceb5* showed the equivalent degree of conidiophore development (Fig.4.6c). Both the *cpr5* (Bowling et al. 1997) and *cpr6* (Clarke et al. 1998) mutants showed a reduction in *P.parasitica* Noco2 conidiophore development in comparison to Col-0, which is comparable to the results obtained here for *ceb1, ceb3, ceb4* and *ceb6*.

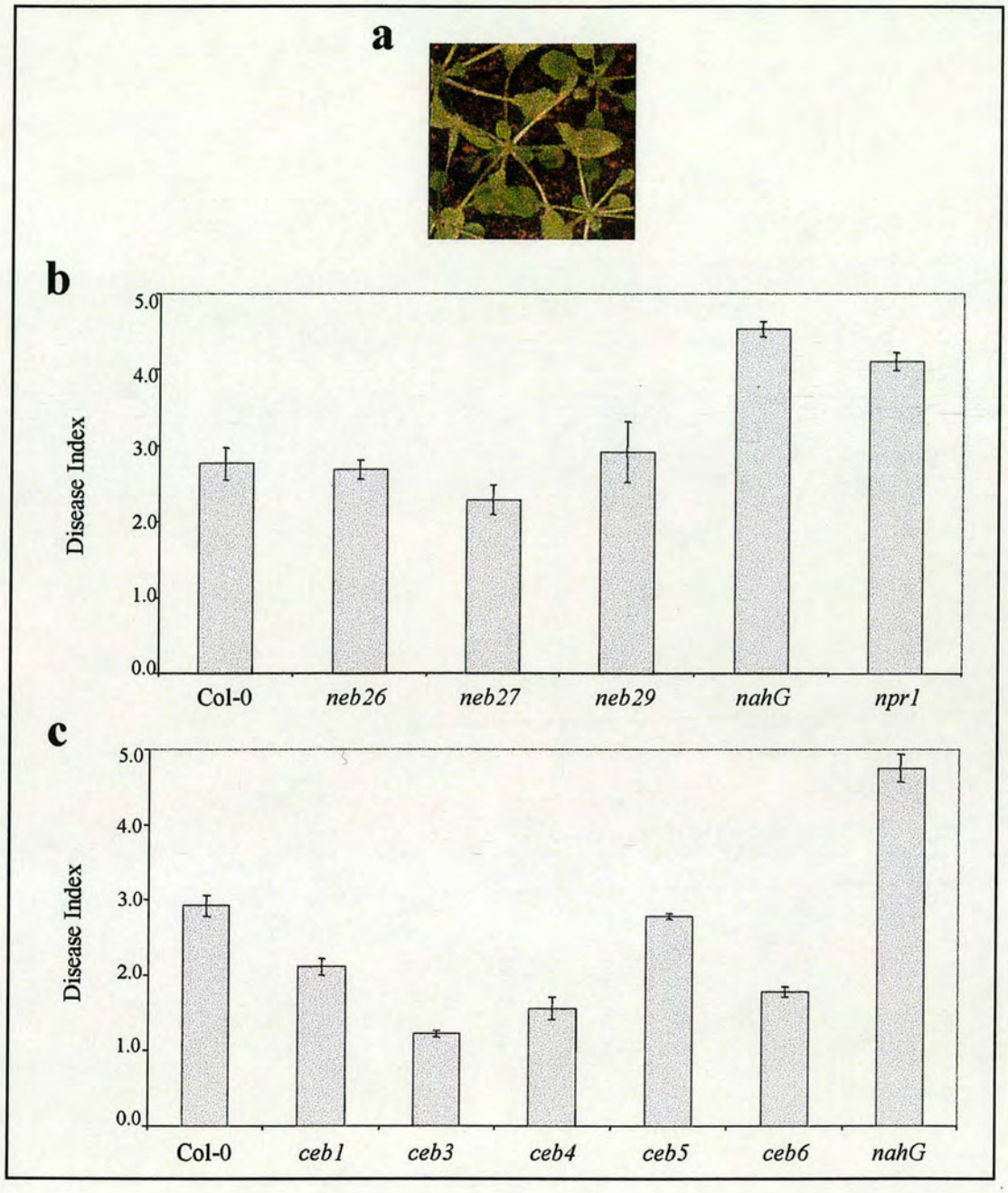

Fig.4.6. Resistance of *neb* and *ceb* candidate mutants to *P.parasitica* Noco2.

a: Downy mildew symptoms on Col-0 10 days after *P.parasitica* Noco2 inoculation.
b: Disease rating of *P.parasitica* Noco2 infection 10 days after inoculation. Col-0, *nahG*, *npr1* and three *neb* candidate mutants were tested.
c: Disease rating of *P.parasitica* Noco2 infection 10 days after inoculation. Col-0, *nahG* and all five c*eb* candidate mutants were tested.
Scoring criteria are outlined in Chapter 2. Four plants were analysed per replicate and three disease indices representing three replicates were obtained per line. Error bars represent the standard error between replicates at the 95% confidence level. Experiments were repeated twice and results obtained followed a similar trend.

Fusarium oxysporum f.sp. *matthiolae*

The soil-borne fungus *F. oxysporum* is the causal organism of vascular wilt in a wide range of plants, including many economically important crops (Agrios 1997). The fungus normally invades the roots (or leaves if spray-inoculated) and colonises the vascular tissue, causing typical symptoms: the leaves turn yellow, wilt and finally die (Fig.4.7a). The interaction between Arabidopsis and *F. oxysporum* f.sp. *matthiolae* has been studied in some detail, and resistant and susceptible ecotypes have been identified (Mauch-Mani&Slusarenko 1994, Epple et al 1998). When leaves were spray-inoculated with a *F. oxysporum* f.sp. *matthiolae* spore suspension and disease symptoms (wilted, necrotic leaves) were monitored eight days later, Arabidopsis ecotype Umkirch (UK4) was found to be resistant to infection with very few leaves showing disease symptoms, whereas ecotype Col-0 was susceptible (Epple et al. 1998).

In order to investigate the extent of *F. oxysporum* f.sp. *matthiolae* resistance in the five *ceb* candidate mutants, *ceb* plants were inoculated with *F. oxysporum* f.sp. *matthiolae* together with Col-0 and UK4 plants. Four-week old plants were sprayed-inoculated with a *F. oxysporum* f.sp. *matthiolae* spore suspension and incubated under humid conditions for eight days, after which time the disease symptoms were recorded. The number of necrotic leaves per plant were noted as an indication of disease severity. Plants were grown in three separate trays, with four plants grown in each tray. Results are presented in Fig.4.7b. As expected, UK4 was resistant to *F. oxysporum* f.sp. *matthiolae* inoculation, whereas Col-0 was susceptible. An average of 1.4% of leaves per UK4 plant wilted and died, whereas 19.9% of Col-0 leaves per plant died (Fig.4.7b). This amounted to a significant difference using the Mann-Whitney test at the 95% confidence level. The *ceb* lines showed a range of responses in comparison to Col-0. The *ceb3, ceb4* and *ceb6* lines had fewer necrotic leaves per plant in comparison to Col-0 which were not significantly different, whereas *ceb1* displayed a similar degree of symptom development in comparison to Col-0 (Fig.4.7b). The *ceb5* plants showed significantly enhanced susceptibility to *F.oxysporum* f.sp. *matthiolae* in comparison to Col-0 (Fig.4.7b).

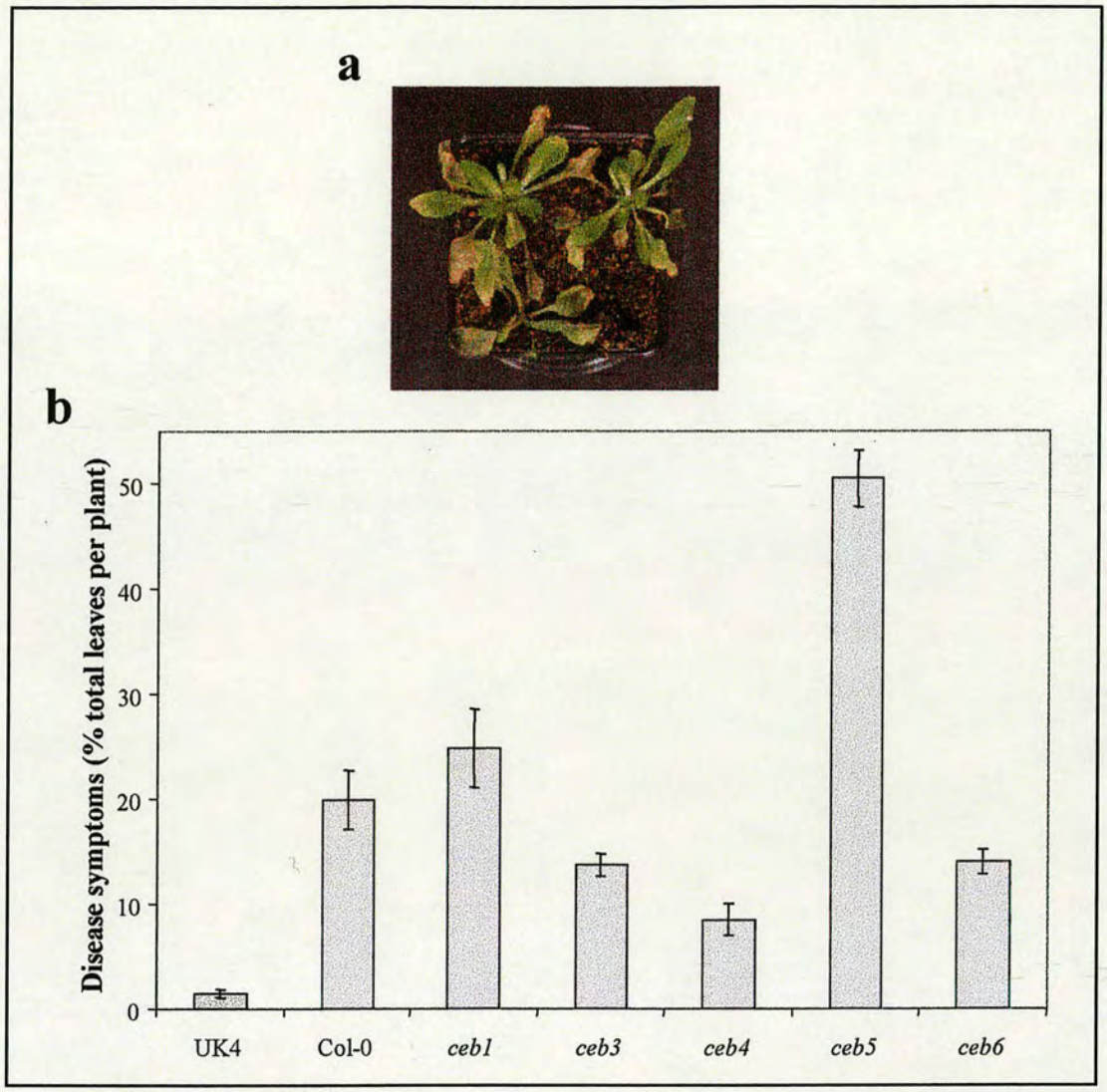

Fig.4.7. Resistance of *ceb* candidate mutants to *F. oxysporum* f.sp. *matthiolae*.

a: Col-0 plant showing typical *F. oxysporum* f.sp. *matthiolae* symptoms eight days after spray-inoculation with a *F. oxysporum* f.sp. *matthiolae* spore suspension.
b: Graph indicating extent of symptom development (percentage of leaves per plant showing *F. oxysporum* f.sp. *matthiolae* symptoms). Col-0, UK4 and the five *ceb* candidate mutants were tested. Values presented are the averages of disease symptoms obtained for 12 plants. Error bars represent the standard error between replicates at the 95% confidence level. The experiment was repeated twice and results obtained followed a similar trend.

4.5. Segregation analysis

The *ceb* candidate mutants were back-crossed to the *PR-1a:luc* transgenic line in order to determine the dominant/recessive nature of the putative mutations (Koorneef & Stam 1992). Pollen was taken from the *ceb* donor plants and applied to pistils of

dissected *PR-1a:luc* flowers, which acted as the pollen recipient. F1 seeds were collected. Five-week old F1 plants were analysed for constitutive luc activity using the ultra low-light imaging camera. The results obtained are outlined in Table 4.1. For the *ceb1, ceb3, ceb4* and *ceb6* crosses, analysis of the F1 plants showed loss of constitutive *PR-1a:luc* expression in all cases, indicating that they define recessive mutations (Table 4.1). On the other hand, all 12 F1 progeny from the *ceb5* X *PR-1a:luc* cross showed constitutive luc activity, indicating that *ceb5* defines a putative dominant mutation (Table 4.1).

Table 4.1. Segregation analysis of the *ceb* candidate mutants.

Generation	Donor	Recipient	No. tested	Luc activity	No luc activity
F1	*ceb1*	*PR-1a:luc*	12	0	12
F1	*ceb 3*	*PR-1a:luc*	12	0	12
F1	*ceb 4*	*PR-1a:luc*	12	0	12
F1	*ceb 5*	*PR-1a:luc*	12	12	0
F1	*ceb 6*	*PR-1a:luc*	12	0	12

4.6. Discussion

The *neb* and *ceb* candidate mutants were tested further in order to determine rigorously if they were true SAR mutants. Expression of defence-related genes and resistance to a range of virulent pathogens is usually used to determine the status of a potential mutant (Cao et al. 1994, Bowling et al. 1994, Clarke et al. 1998) and these criteria were applied to *neb* and *ceb* candidates.

After inoculation with *Pst*DC3000 *(avrB)*, the four *neb* candidate mutants were shown to express a reduced amount of *PR-1a:luc* in comparison to wildtype *PR-1a:luc* transgenic plants (Fig.4.1a&b). SA application induced luc activity in the *neb* plants (Fig.4.1c), indicating that they may define a mutant class with a block upstream of SA in the signal transduction pathway leading to SAR. However, the *neb* lines showed expression of *PR-1* following *Pst*DC3000 *(avrB)* in Northern blot

analysis (Fig.4.3). It is unlikely that this discrepancy in luminometer and Northern blot results can be explained by loss of the *PR-1a:luc* transformation cassette in the *neb* lines. The *neb* candidates were tested during the secondary phase of the mutant screen for sensitivity to kanamycin during seedling germination and all the lines were resistant. It is also unlikely that the *PR-1a-luc* sequence was mutagenised in these lines, or that silencing of the *PR-1a* promoter by methylation (Finnegan & McElroy 1994) has occurred as SA induced *PR-1a:luc* expression in all cases. It is possible that the *neb* candidates represent loss of upstream signalling components required for transcription of the tobacco *PR-1a* gene which are not required for the transcription of *PR-1* in Arabidopsis. Consistent with this possibility, the *neb* plants tested here did not show a significant difference in resistance to *Pst*DC3000 or *P.parasitica* Noco2 in comparison to Col-0, and thus can not be considered to be SAR mutants. For this reason it was decided to do no further testing of this *neb* candidate mutant class and to concentrate on characterising the *ceb* candidate mutants.

All five *ceb* candidate mutants expressed the SA-dependent defence genes *PR-1, PR-2* and *PR-5* constitutively in naïve plants (Fig.4.4a&b), indicating that they represent mutations in the SA-dependent signal transduction pathway leading to expression of these genes (Ryals et al. 1996). These mutations may be either up- or downstream of SA. Of the five *ceb* lines, *ceb3* and *ceb5* expressed *PR-1* to levels higher than Col-0 plants treated with SA, indicating that the pathway in these lines is very strongly induced. The *PR-1* Northern blot result for *ceb5* corresponded to the luminometer data (Fig.4.2) as luc activity in *ceb5* increased in comparison to *PR-1a:luc* plants treated with SA (Fig.4.2). The *ceb1* line shows the greatest increase in luc activity in comparison to SA-treated *PR-1a:luc* plants (Fig.4.2), but only showed a moderate increase in *PR-1* expression (Fig.4.4a). In this respect, *ceb1* is analogous to *cpr5*, which showed a two-fold increase in GUS activity in comparison wild-type plants treated with INA, whereas constitutive *PR-1* expression was significantly less than in wild-type plants treated with INA (Bowling et al. 1997).

Expression of the *PDF1.2* gene is dependent on both jasmonate and ethylene signalling (Penninclx et al. 1998). All five *ceb* candidate mutants expressed this gene constitutively (Fig.4.4a), indicating that they represent mutations in the jasmonate and ethylene signal transduction pathway leading to *PDF1.2* expression. However, all five *ceb* plants did not express *Thi2.1* and only *ceb5* showed a moderate increase in *AtLOX2* expression (Fig.4.4c&d). Expression of the *Thi2.1* gene is dependent on jasmonate signalling (Bohlmann et al. 1998). Although not shown in Fig.4.4d, expression of *AtLOX2* was previously found to be responsive to Me-JA application (Bell & Mullet 1993), indicating that only *ceb5* may contain higher levels of methyl jasmonate or other jasmonate signalling components. Expression of *ACS2* is not upregulated in any of the *ceb* plants (Fig.4.4c), suggesting that ethylene levels may not be altered in *ceb* plants in comparison to wild-type. ACS2 is the major isoform of ACC synthase in Arabidopsis and wound-induced expression of *ACS2* is controlled at the level of transcription (Liang et al. 1992). However, recently it has been reported that ethylene over-production may be controlled by post-transcriptional regulation of ACS isoforms under different conditions (Woeste et al. 1999a, Woeste et al. 1999b). This suggests that a lack of up-regulation in *ACS2* expression does not necessarily indicate that ethylene production is not increased in the *ceb* plants. Thus, although expression of genes involved in the jasmonate and ethylene response is not clear, it is interesting that all five *ceb* lines showed constitutive expression of both *PR-1* and *PDF1.2* and thus may define mutations common to both SA, jasmonate and ethylene signal transduction.

None of the five *ceb* candidate mutants developed macroscopic lesions spontaneously, but all expressed *GST1* constitutively at low levels (Fig.4.4b). Expression of *GST1* can be used to report the accumulation of ROS (Grant & Loake 2000, in press). Thus, it is likely that the *ceb* candidates accumulate low levels of ROS, or that GSTs are playing an alternative role in removing potentially toxic signalling components accumulated due to constitutive activation of SAR.

In the resistance assay to the virulent bacterial pathogen *Pst*DC3000, only *ceb1* and *ceb6* showed a significant reduction in bacterial growth in comparison to Col-0

(Fig.4.5c). In the resistance assay to the virulent oomycete pathogen *P.parasitica* Noco2, *ceb1, ceb3, ceb4* and *ceb6* showed a significant reduction in conidiophore development in comparison to Col-0 (Fig.4.6c). None of the *ceb* plants showed increased resistance to the necrotrophic fungal pathogen *F.oxysporum* f.sp.*matthiolae*, and *ceb5* showed significantly enhanced susceptibility to the fungus in comparison to Col-0 (Fig.4.8). This result corresponded to the lack of constitutive *Thi2.1* expression in all the *ceb* lines (Fig.4.4c), as resistance to *F.oxysporum* f.sp.*matthiolae* in Arabidopsis is associated with increased expression of *Thi2.1* (Epple et al. 1998).

It is interesting to note that *ceb5*, which shows the strongest expression of defence genes in Northern blot analysis (Fig.4.4a,b,c,d), also displayed earlier senescence in comparison to wild-type Col-0 plants (results not shown). Recently it has been shown that *PR-1* is expressed in senescing Arabidopsis plants and that *PR-1* expression under these conditions is dependent on SA signal transduction (Morris et al. 2000). Thus, it is possible that *ceb5* defines a gene important in SA-dependent senescence signal transduction. *ceb5* plants displayed no significant increase in disease resistance to *Pst*DC3000 (Fig.4.5c) or *P.parasitica* Noco2 (Fig.4.6c). It is likely that other defence genes, which have yet to be discovered and are required for resistance to these two pathogens, have not been induced in *ceb5*. Furthermore, *ceb5* was more susceptible to *F.oxysporum* f.sp. *matthiolae*, indicating that induction of *PR-1, PR-2, PR-5, PDF1.2* and *GST1* expression and possibly other unidentified genes may result in 'cross-talk' and suppression of elements involved in resistance to *F.oxysporum* f.sp. *matthiolae.* Alternatively, the early senescence phenotype of *ceb5*, resulting in enhanced necrosis in this line, may promote colonisation of the plant by *F.oxysporum* f.sp. *matthiolae*, a necrotrophic fungal pathogen. Recently it has been shown that *Botrytis cinerea*, also a necrotrophic fungal pathogen, triggered cell death and the production of an HR in Arabidopsis, thereby facilitating its colonisation of plants (Govrin & Levine 2000). It is possible that *F.oxysporum* f.sp. *matthiolae* uses a similar mechanism in pathogenesis, which is enhanced by increased cell death in *ceb5*. Thus, although *ceb5* has an interesting phenotype and bears further investigation, the aim of the current study was to characterise SAR

mutants showing enhanced disease resistance and no further characterisation of *ceb5* was performed.

Taking the expression of defence-related genes and disease resistance assays into account, it was decided to select *ceb1* for further characterisation. Luc activity in *ceb1* was high, the defence-related genes *PR-1, PR-2, PR-5, PDF1.2* and *GST1* were expressed constitutively and *ceb1* plants were significantly more resistant than wild-type Col-0 plants to both a virulent bacterial and oomycete pathogen. For these reasons *ceb1* can be considered to be a true SAR mutant and it was decided to re-name this candidate *c*onstitutively *i*nduced *r*esistance *1* (*cir1*). Further detailed characterisation of *cir1* will be presented in Chapter 5.

Chapter Five

Further characterisation of *cir1*

5.1. Introduction

The *cir1* mutant was selected from the group of *ceb* putative mutants for further characterisation. The SA-dependent defence related genes *PR-1, PR-2* and *PR-5* and the jasmonate- and ethylene-dependent gene *PDF1.2* are expressed constitutively in *cir1*. In addition, *cir1* is resistant to both a virulent bacterial pathogen, *Pst*DC3000, and a virulent oomycete pathogen *P.parasitica* Noco2. Preliminary genetic analysis indicated that *cir1* is a recessive mutation.

In this chapter, a more comprehensive analysis was made of the phenotype of *cir1*. Further segregation analyses were conducted to confirm the recessive nature of *cir1*. Complementation analyses of *cir1* to other mutants displaying constitutive SAR were also conducted. The map position of *cir1* was determined and the relationship of *cir1* to SA and ethylene was investigated. The position of *cir1* in the SAR signal transduction network was further investigated by creating double mutants between *cir1* and *npr1, ein2.1* and *jar1*. These double mutants were analysed for constitutive luc activity, constitutive expression of *PR-1* and *PDF1.2*, and enhanced resistance to *Pst*DC3000 and *P.parasitica* Noco2.

5.2. Visual phenotype of *cir1*

Soil-grown *PR-1a:luc* Col-0 and *cir1* plants were routinely grown under short day conditions (growth conditions described in Chapter 2) and analysed at 5 weeks after planting, at which time leaves were large and fully expanded. Plants were analysed by imaging luc activity in the ultra low-light imaging camera. Maximal constitutive luc activity was obtained in five-week old naïve *cir1* plants whereas no light was produced in *PR-1a:luc* transgenic Col-0 plants of the same age (Fig.5.1a). In comparison naïve *cir1* seedlings grown *in vitro* did not show constitutive *PR-1a:luc* expression (data not shown).

Upon initial visual observation, *cir1* mutant plants did not appear to show any phenotypic differences to *PR-1a:luc* Col-0 plants. In order to record the growth features of *cir1* in comparison to Col-0, ten plants of both *cir1* and *PR-1a:luc* Col-0 were grown under the same conditions. The diameter of the rosette from each five-week plant was measured (Table 5.1) and representative plants were photographed (Fig.5.1b). At this point, the plants were transferred to long-day conditions and the number of days to bolting was recorded (Table 5.1). A plant was considered bolted when the stem was approximately 2cm in length. The bolt height of each plant was recorded at 8 weeks (Table 5.1).

The *cir1* mutant plants were slightly smaller than *PR-1a:luc* Col-0 in terms of both rosette size and bolt height, and bolting in *cir1* was initiated slightly later than in Col-0 (Table 5.1). Leaves of *cir1* plants had a slightly longer, thinner shape in comparison to *PR-1a:luc* Col-0 (Fig.5.1.b).

Table 5.1. Growth features of *cir1* in comparison to *PR-1a:luc* Col-0 plants.

	PR-1a:luc Col-0	*cir1*
Rosette diameter (Day 35)	6.71 (0.07)	4.91 (0.05)
Days to bolting	45	47-49
Bolt height (Day 56)	32.94 (1.61)	26.69 (2.29)

Measurements are shown in centimetres (cm), with the standard error at the 95% confidence level shown in parentheses. Ten plants of both *cir1* and Col-0 were analysed.

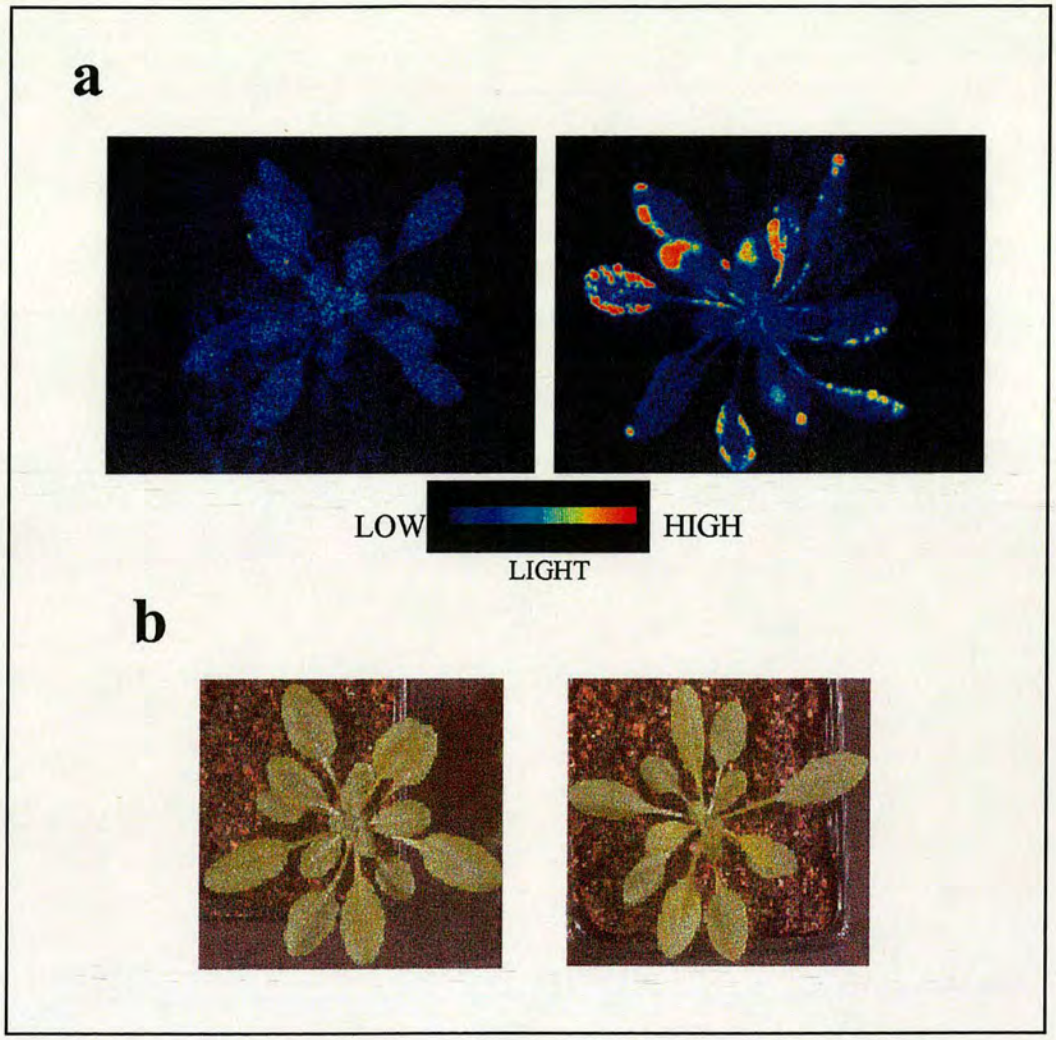

Fig.5.1. The phenotype of *cir1* in comparison to *PR-1a:luc* Col-0 plants.
a: Five-week old representative *PR-1a:luc* and *cir1* plants were painted with 1mM luciferin. Plants were imaged for constitutive luc activity in the ultra low-light imaging camera. *PR-1a:luc* is shown on the left, *cir1* on the right.
b: Representative five-week old *PR-1a:luc* Col-0 and *cir1* plants. *PR-1a:luc* is shown on the left, *cir1* on the right.

As shown in Fig.5.1b, no spontaneous HR lesion formation was visualised in *cir1* leaves. However, it has been reported that micro-lesion formation, as a result of accumulating ROS, underlies SAR (Alvarez et al. 1998) and thus micro-lesion formation in *cir1* may be occurring. In order to test this, leaves from four-week old naïve *cir1* plants were stained with trypan blue (Cao et al. 1998). Trypan blue stains dead cells thus allowing for easy identification of potential micro-lesions. Of the ten *cir1* leaves stained, no areas of dead cells could be visualised as was the case with Col-0 leaves. Trypan blue staining revealed areas of dead cells in leaves from the

cpr5 mutant. The *cpr5* mutant shows spontaneous formation of lesions and a similar pattern of constitutive defence gene expression and disease resistance as *cir1* (Bowling et al. 1997). A representative trypan blue stained *cir1* leaf is shown in Fig.5.2 and a *cpr5* leaf is shown as the positive control.

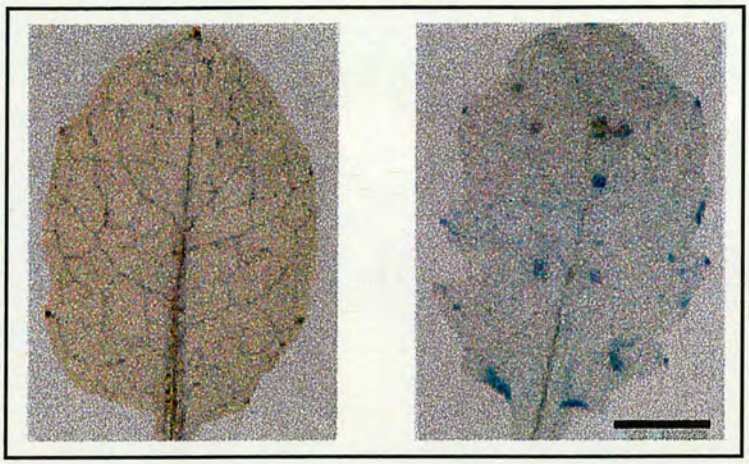

Fig.5.2. Comparison of micro-lesion formation between *cir1* and *cpr5*.
Leaves were stained with trypan blue, de-stained and areas of cell death were visualised using a dissecting microscope. Dead cells were visualised as areas with a dark blue colour. *cir1* is shown on the left, *cpr5* is shown on the right. Size bar represents 5mm.

These results show that the only easily identifiable phenotype of *cir1* was constitutive expression of *PR-1a:luc* in five-week old naïve plants. This phenotype was thus chosen as the method of identifying *cir1* in the progeny of crosses described in the following section.

5.3. Genetic analysis of *cir1*

Segregation analysis

As recorded in Chapter 4, the *ceb* mutant lines were back-crossed to the *PR-1a:luc* Col-0 transgenic line. Loss of *PR-1a:luc* expression was detected in the F1 progeny of the *cir1* X *PR-1a:luc* cross, indicating that *cir1* is a recessive mutation (Koorneef & Stam 1992). In order to confirm this result, reciprocal crosses were made between *cir1* and *PR-1a:luc* Col-0 and between *cir1* and Col-0. Five-week old plants from both the F1 and the F2 progeny were analysed for constitutive luc activity using the ultra low-light imaging camera. The results obtained are outlined in Table 5.2.

Analysis of the F1 plants showed loss of constitutive luc activity in all cases, confirming that *cir1* is a recessive mutation. To determine if *cir1* defines a single gene, the segregation of *PR-1a:luc* expression was investigated in F2 populations of both types of crosses. In the case of the F2 population of the *cir1* X *PR-1a:luc* Col-0 cross, a ratio of one plant with luc activity to three plants without luc activity was obtained, indicating that the *cir1* trait is controlled by a single gene and that the mutated *CIR1* gene is not linked to the *PR-1a:luc* transgene. This result was confirmed by analysis of the F2 progeny of the *cir1* X Col-0 cross. However, in this case, the *PR-1a:luc* transgene segregates as a monogenic dominant trait and constitutive luc activity was only visualised in three-quarters of the F2 plants homozygous for *cir1* (Table 5.2).

Complementation analysis

As documented in Chapter 4, a total of four recessive mutations displaying constitutive SAR were isolated in this study. In order to determine if *cir1* is allelic to any of the other three mutants, crosses were made between *cir1* and *ceb3, ceb4* or *ceb6*. In addition, *cir1* was crossed to both the *cpr1* (Bowling et al. 1994) and *cpr5* (Bowling et al. 1997) mutants, which are also recessive and display constitutive SAR. *cpr1* has been placed downstream of the HR, but upstream of SA accumulation in the SAR signal transduction pathway (Bowling et al. 1994, Fig.1.3), whereas *cpr5* has been placed upstream of the HR (Bowling et al. 1997, Fig.1.3). F1 plants from each cross were imaged using the ultra low-light imaging camera in order to identify plants producing constitutive luc activity. The rationale was that the presence of a single copy of the *PR-1a:luc* transgene would be sufficient to identify constitutive *PR-1a:luc* expression, if any of the recessive mutations were allelic to *cir1* and thus unable to complement *cir1* in F1 plants. The results obtained are outlined in Table 5.3. Constitutive luc activity was lost in the majority of the F1 progeny of all the crosses, except for the progeny of the *cir1* X *ceb3* cross, all of which showed constitutive luc activity. This indicated that *cir1* and *ceb3* were allelic. Although the reciprocal cross was not performed, it is unlikely that the constitutive *PR-1a:luc* expression in F1 plants is simply due to self-pollination of

Table 5.2 Segregation analysis of *cir1*.

Generation	Donor	Recipient	Hypothesis	No. tested	Luc activity		No luc activity		χ^2	*P=5%*	Accept?
					Observed	Expected	Observed	Expected			
F1	*PR-1a:luc*	*cir1*		12	0	0	12	12			
F1	*cir1*	*PR-1a:luc*		12	0	0	12	12			
F2	*cir1*	*PR-1a:luc*	1:3[a]	60	15	15	45	45	0	<3.841	Yes
F1	Col-0	*cir1*		16	0	0	16	16			
F1	*cir1*	Col-0		16 *	0	0	16	16			
F2	*cir1*	Col-0	3:13[b]	72	10	13.5	62	58.5	1.116	<3.841	Yes

The χ^2 value was calculated and compared to the critical value at the 5% probability level (Ennos 2000).

[a] The hypothesis was that *cir1* segregates as a monogenic recessive trait with respect to constitutive luc activity.
[b] The hypothesis was that *cir1* segregates as a monogenic recessive trait. However, the *PR-1a:luc* transgene will segregate as a monogenic dominant trait and constitutive luc activity will only be visualised in three-quarters of the F2 plants containing the *cir1* mutation.
* These F1 plants were identified as kanamycin resistant *in vitro*, thus indicating that the transformation cassette containing the *PR-1a:luc* gene and the kanamycin resistance gene had been donated by the *cir1* pollen donor.

ceb3, as the 20 F1 plants analysed originated from a pool of seed from three separate silliques. The F2 progeny of *cir1* X *ceb3* cross was analysed for constitutive luc activity and 53 plants out of 54 showed constitutive luminescence (Table 5.3), further indicating that *cir1* and *ceb3* were allelic. It is likely that the single plant not showing constitutive *PR-1a:luc* expression was due to contamination during seed collection. Twelve F1 plants from the *cir1* X *cpr1* cross were examined for constitutive luc activity. One plant was found to express *PR-1a:luc* constitutively (Table 5.3) and the reason for this unexpected segregation is unknown. However, it appears that *cir1* is not allelic to either *cpr1* or *cpr5*.

Table 5.3. Complementation analyses of *cir1*.

Generation	Donor	Recipient	No. tested	Luc activity	No luc activity
F1	*cir1*	*ceb3*	20	20	0
F2	*cir1*	*ceb3*	54	53	1
F1	*cir1*	*ceb4*	24	0	24
F1	*cir1*	*ceb6*	20	0	20
F1	*cir1*	*cpr1*	12	1	11
F1	*cir1*	*cpr5*	3	0	3

Mapping cir1

In order to determine the map position of the *cir1* mutation, *cir1* Col-0 plants were crossed to Landsberg erecta (L*er*) plants. The F1 progeny did not exhibit constitutive *PR-1a:luc* expression as expected for a recessive trait (data not shown). These plants were allowed to self-pollinate and the F2 progeny of F1 plants were analysed for constitutive *PR-1a:luc* expression using an ultra low-light imaging camera. Initially an F2 population of 62 plants with constitutive luc activity were identified. The chromosomal location of the *cir1* mutation was then mapped with the 62 plants and a variety of PCR-based markers. Linkage analysis was performed by amplification of the polymorphic DNA regions corresponding to the marker, followed by the determination of the PCR product sizes. CAPS (cleaved co-dominant amplified polymorphic sequences; Konieczny & Ausabel 1993) and SSLP (single sequence length polymorphism; Bell & Ecker 1994) markers approximately

20 to 30 centimorgans (cM) apart on the recombinant inbred genetic map (Lister & Dean 1993) were used. Markers used in this study were chosen from each of the five chromosomes and are outlined in Table 5.4. Markers were selected from databases maintained at TAIR (http://www.arabidopsis.org) and were chosen for easy identification of the polymorphic Col-0 and L*er* PCR products by agarose gel electrophoresis. Because the markers are co-dominant, the genotype for all 124 chromosomes could be monitored and scored at every locus tested. Generally, a recombination frequency of less than 30% in a small population is accepted as evidence for linkage between the mutation and the marker under test (Ponce et al. 1999). However, linkage could not be identified between any of the markers tested in this population of 62 plants (results not shown). It is likely that this was due to mis-scoring of the *cir1* mutant phenotype (constitutive luc activity as scored in the ultra low-light imaging camera) and *PR-1a:luc* plants were scored as *cir1*. Mis-scoring of the mutant phenotype is thought to be a common problem in mapping experiments (Lukowitz et al. 2000).

In order to solve this problem, a different F2 population from the *cir1* X L*er* cross was screened and a further 24 F2 plants producing constitutive luc activity were identified. Greater care was taken to ensure that the plants were correctly scored. All plants were grown under uniform growth conditions and plants were scored for constitutive luc activity when they were exactly five weeks old. Positive controls (*cir1* plants) were grown in each tray and the number of leaves showing constitutive *PR-1a:luc* expression per plant were determined. Generally, five-week old *cir1* plants have approximately one half of all leaves expressing *PR-1a:luc* constitutively (Fig.5.1a). F2 plants were only scored for the mutant phenotype if they corresponded exactly to *cir1* in the tray undergoing analysis, namely that one half of all leaves produced constitutive luc activity. Using the 48 chromosomes contributed by these 24 plants and the PCR-based markers outlined in Table 5.4, linkage was established to markers on the lower arm of chromosome 4 (Table 5.4). In order to get a clearer idea of the map position of *cir1* on chromosome 4, a further 24 F2 plants expressing constitutive luc activity were identified and analysed for linkage to markers on chromosome 4 (Table 5.4). Data from these 96 chromosomes indicated

Table 5.4. Mapping *cir1* using CAPS and SSLP markers.

		Chromosome 1			Chromosome2		Chromosome3		Chromosome4						Chromosome5			
		nga63	AthSO392	nga128	THY1	nga168	ArLIM15	nga707	GA1	nga8	nga1111	g4539	AG	RPS2	nga1107	nga151	AtSO262	AthSO191
Plant Number[a]		**9.3[b]**	**46**	**100**	**30**	**73**	**30**	**75**	**17.7**	**26.5**	**29.6**	**57.6**	**63.1**	**75.7**	**102**	**26**	**62**	**103**
1	1a	C	L	L	L	L	H	U	H	H	H	U	C	C	H	C	H	H
2	1c	L	L	H	H	L	H	U	H	H	C	U	C	C	C	H	H	H
3	4a	C	C	H	H	C	C	U	H	H	H	U	H	L	L	H	H	H
4	5c	H	H	H	L	H	H	C	C	C	C	C	C	C	C	H	H	H
5	6b	C	C	L	L	L	C	H	C	C	C	C	C	C	C	L	L	L
6	12d	H	L	C	H	H	L	L	H	L	L	L	L	H	H	L	L	L
7	13b	U	L	H	H	L	H	C	C	C	C	C	C	H	H	L	H	H
8	13d	L	L	C	U	C	L	L	C	C	C	C	C	U	H	L	H	H
9	14d	C	C	L	C	H	H	C	H	H	H	H	H	H	H	H	H	H
10	15c	C	C	U	H	H	H	U	C	C	C	U	C	H	C	H	H	H
11	15d	U	H	L	H	H	L	H	C	C	C	C	C	C	C	L	L	L
12	1a	L	L	C	C	H	H	C	C	C	C	C	C	C	C	L	H	H
13	2a	C	C	H	H	H	H	C	C	C	C	C	C	C	L	H	H	H
14	9d	C	H	H	C	C	C	U	C	C	C	U	U	H	C	U	C	C
15	10d	L	L	H	L	L	H	U	C	C	C	U	H	C	H	L	H	H
16	11b	U	L	H	C	H	C	U	U	C	C	U	U	C	L	C	H	H
17	11d	H	H	H	C	H	C	C	C	C	C	C	C	C	H	L	C	C
18	16a	H	L	L	L	L	H	L	U	C	C	C	C	H	H	H	H	H
19	16b	U	H	H	H	H	H	U	H	C	C	U	C	C	C	C	H	H
20	17d	H	H	H	H	H	C	C	C	C	C	H	H	C	H	L	L	L
21	18c	C	C	H	L	H	C	U	C	C	C	U	H	H	H	U	H	H
22	21d	U	C	C	U	C	C	U	U	C	C	U	U	C	C	U	H	H
23	26d	U	L	C	H	H	H	U	C	C	C	U	C	U	H	C	H	H
24	27a	U	L	U	U	L	L	U	U	C	C	U	U	C	C	U	H	H
25	3b								C	C	C	C	C	H				
26	4d								U	C	C	U	U	C				
27	6c								C	C	C	C	C	C				

(continued on next page)

Table 5.4. *(continued from previous page)*

		Chromosome1			Chromosome2		Chromosome3		Chromosome4						Chromosome5			
		nga63	AthSO392	nga128	THY1	nga168	ArLIM15	nga707	GA1	nga8	nga1111	g4539	AG	RPS2	nga1107	nga151	AtSO262	AthSO191
Plant Number[a]		**9.3[b]**	**46**	**100**	**30**	**73**	**30**	**75**	**17.7**	**26.5**	**29.6**	**57.6**	**63.1**	**75.7**	**102**	**26**	**62**	**103**
28	7b								C	C	C	C	C	C				
29	11c								C	C	C	C	H	H				
30	13a								H	H	H	C	C	C				
31	13c								L	H	H	C	U	C				
32	26c								H	H	C	C	C	C				
33	27d								C	C	C	U	C	C				
34	28c								C	C	C	H	H	L				
35	29a								H	C	C	C	C	C				
36	29d								H	C	C	C	C	C				
37	1d								H	H	H	H	H	H				
38	3c								C	C	C	C	H	H				
39	4d								C	C	C	C	C	C				
40	8c								C	C	C	C	C	H				
41	15a								C	C	C	U	C	H				
42	16c								H	H	C	C	C	C				
43	18c								H	C	C	U	H	H				
44	23d								C	C	C	C	C	H				
45	24d								C	C	C	C	H	H				
46	28a								C	C	C	H	H	H				
47	29c								H	H	H	H	H	L				
48	30a								U	C	C	C	C	C				
%R[c]		40.6	58.3	50	52	56.25	41.6	33.3	17.4	12.5	9.4	12.5	16.6	25	35.4	62.5	54	54

[a] All 48 F2 plants analysed segregated as a recessive trait and exhibited the *cir1* phenotype of constitutive luc activity. Plants were analysed in two separate groups: 1 to 24 and 25 to 48.

[b] Genetic position on the recombinant inbred (RI) map (Lister & Dean 1993). Position is indicated in centimorgans (cM).

[c] %R = Percentage recombination, calculated as follows: 2 X L scores + H scores / total number of chromosomes scored.

C = homozygous Col-0, H = heterozygous, L = homozygous L*er*, U = Undetermined

that *cir1* maps to a 28 cM region, approximately 9.4 cM south from nga1111 and approximately 12.5 cM north from g4539 (Table 5.4).

5.4. Relationship of *cir1* to SA

Treatment of a wide variety of plants with SA is known to induce *PR-1* gene expression (Ryals et al. 1996) and accumulation of SA often precedes or parallels *PR-1* gene induction during SAR in Arabidopsis (Cameron et al. 1999). Having shown that *cir1* displayed constitutive expression of *PR-1, PR-2* and *PR-5*, it was necessary to determine the position of *cir1* relative to SA in the SAR signal transduction pathway. Both a biochemical and a genetical approach were taken in order to investigate the relationship of *cir1* to SA.

Determination of the endogenous level of SA in cir1

The amount of endogenous SA in five-week old naïve Col-0 and *cir1* plants was determined by HPLC analysis (Thomson, unpublished results). Col-0 plants inoculated with *Pst* DC3000 *(avrB)* two days before analysis were included as a control. As shown in Fig.5.3, levels of endogenous SA in *cir1* plants was approximately ten times higher than Col-0. Interestingly, endogenous SA levels in *cir1* were approximately the same as the level of SA accumulated in Col-0 plants inoculated with *Pst* DC3000 *(avrB)*.

The cir1 phenotype in nahG plants.

To define the role of SA in *cir1* plants, a cross was made between *cir1* and a transgenic plant containing the *nahG* gene from *Pseudomonas putida* (Delaney et al. 1994). *nahG* encodes a salicylate hydroxylase which converts SA to catechol. Plants expressing the *nahG* transgene are thus unable to accumulate SA, undergo SAR or express *PR-1* in response to avirulent pathogens (Lawton et al. 1995). As *cir1* segregates as a recessive trait, F1 plants from the *cir1* X *nahG* cross did not express constitutive *PR-1a:luc* (Table 5.5). F1 plants were allowed to self-pollinate and F2 plants were analysed for constitutive *PR-1a:luc* expression. As *cir1* segregates as a monogenic recessive trait and the *PR-1a:luc* transgene segregates as a

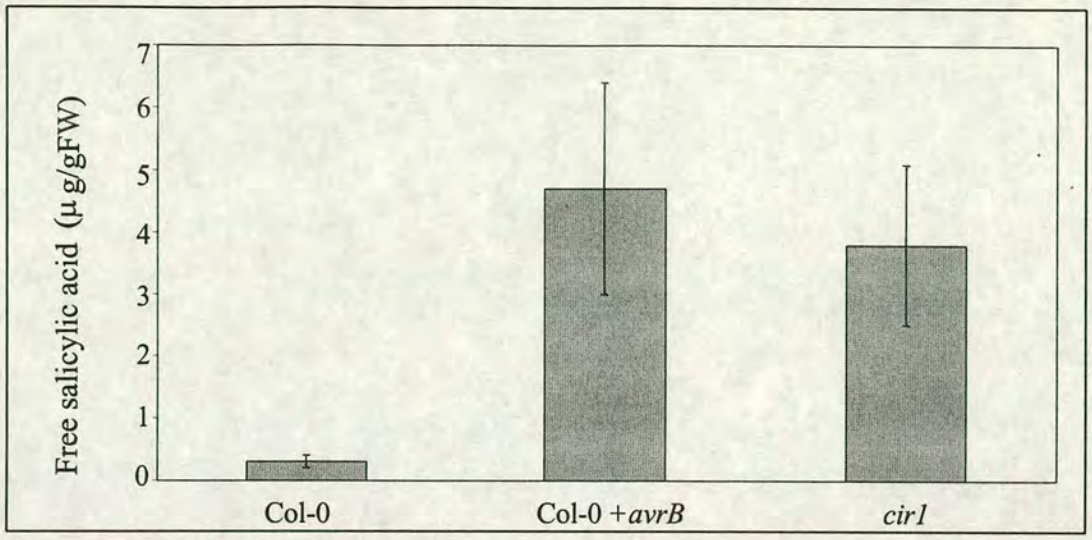

Fig.5.3. Comparison of free SA levels in Col-0, Col-0 inoculated with *Pst* DC3000 *(avrB)* and *cir1*.

Col-0 plants were inoculated with *Pst*DC3000 (*avrB*) two days prior to analysis. Leaves from five-week old plants were collected for HPLC analysis of free SA content. The values presented are the average of three replicates ± SE (micrograms SA per gram fresh weight leaf tissue).

monogenic dominant trait, constitutive luc activity will normally only be visualised in three-quarters of the F2 plants containing the *cir1* mutation. In this case the hypothesis was that *nahG*, which segregates as a monogenic dominant trait, will degrade SA in three quarters of the F2 plants, leading to loss of constitutive luc activity in three quarters of the plants. Nineteen F2 plants were analysed, two of which produced constitutive luc activity (Table 5.5). This was consistent with the ratio of 3 plants expressing *PR-1a:luc* to 61 plants not showing luc activity (Table 5.5). F2 plants were allowed to self-pollinate and F3 seedlings were tested for sensitivity to SA *in vitro*. When *nahG* plants are grown on 0.5mM SA *in vitro*, brown deposits are formed in the roots (Bowling et al. 1994). These deposits are phenolic by-products from the breakdown of catechol (Bowling et al. 1994). Of the 19 F3 families grown on SA, 12 showed brown deposits in the roots. The two F3 families which produced constitutive luc activity did not show brown deposits in the roots, indicating that the F2 parents did not contain the *nahG* transgene. These results indicate that loss of SA accumulation in *cir1* abolishes constitutive luc expression in *cir1*. As the visual phenotype of *cir1* (constitutive luc activity) is dependent on the accumulation of SA, it was not possible to isolate a *cir1:nahG* double mutant using the ultra low-light imaging camera.

Isolation of a cir1:npr1 double mutant.

The mutant *npr1* is insensitive to SA accumulation and abolishes *PR* gene expression during SAR (Cao et al. 1994). In order to determine if *cir1*-induced constitutive luc activity is dependent on a functional NPR1 protein, a cross was made between *cir1* and *npr1* plants. As both mutants segregate as recessive traits, F1 plants did not produce constitutive luc activity (Table 5.5). F1 plants were allowed to self-pollinate and F2 plants were analysed for constitutive luc activity. As *cir1* segregates as a monogenic recessive trait and the *PR-1a:luc* transgene segregates as a monogenic dominant trait, constitutive luc activity will normally only be visualised in three-quarters of the F2 plants containing the *cir1* mutation. In the case of the F2 population from the *cir1* X *npr1* cross, two hypotheses were possible. Firstly, that *npr1* would abolish constitutive *PR-1* activity, as was the case in the *cpr5:npr1* double mutant (Bowling et al. 1997). Secondly, that *npr1* would not suppress constitutive *PR-1* activity, as was the case in the *cpr6:npr1* double mutant (Clarke et al. 1998). Out of a population of 54 F2 plants from the *cir1* X *npr1* cross, 11 produced constitutive luc activity (Table 5.5). This data fitted both hypotheses (Table 5.5), thus making it difficult to determine the influence of *npr1* on *cir1*-induced constitutive luc activity. The presence of the *npr1* mutation in the F2 population was thus determined. The wild-type *NPR1* gene contains a NIaIII restriction digestion site which is abolished in the *npr1-1* allele (Cao et al. 1997). This was the allele of the *npr1* mutant used in the present study. Thus, restriction digestion analysis of the *npr1-1* region amplified by PCR is a tool that can be used to determine the presence of a homozygous *npr1-1* mutation (Clarke et al. 1998). DNA was extracted from the 11 F2 plants with constitutive luc activity and an additional 16 F2 plants selected at random from the F2 population of 54 plants. Col-0 wild-type and *npr1* plants were also included as controls. PCR with *npr1-1* specific primers was performed on all 29 samples and the PCR products were digested with the NIaIII restriction enzyme. A 100 bp product is produced in Col-0 samples which is missing in homozygous *npr1-1* plants (results not shown). Thus, the 27 F2 plants could be scored for the presence of the *npr1-1* mutation. Out of the 11 F2 plants which showed constitutive luc activity, one contained the homozygous *npr1-1* mutation. This F2 plant was thus a *cir1:npr1* double mutant. Out of the additional

Table 5.5. Genetic analysis of *cir1* X *nahG* and *cir1* X *npr1*

Generation	Donor	Recipient	Hypothesis	No. tested	Luc activity		No luc activity		χ^2	*P=5%*	Accept?
					Observed	Expected	Observed	Expected			
F1	*cir1*	*nahG*		10	0	0	10	10			
F2	*cir1*	*nahG*	3:61[a]	19	2*	1	17	18	1.055	<3.841	Yes
F1	*cir1*	*npr1*		2	0	0	2	2			
F2	*cir1*	*npr1*	9:55[b]	54	11**	8	43	46	1.32	<3.841	Yes
F2	*cir1*	*npr1*	3:13[c]	54	11**	10	43	44	0.12	<3.841	Yes

The χ^2 value was calculated and compared to the critical value at the 5% probability level (Ennos 2000).

[a] *cir1* segregates as a monogenic recessive trait. In addition, the *PR-1a:luc* transgene segregates as a monogenic dominant trait and normally constitutive luc activity will only be visualised in three-quarters of the F2 plants containing the *cir1* mutation. In this case the hypothesis was that *nahG*, which segregates as a monogenic dominant trait, will degrade SA in three quarters of the F2 plants, leading to loss of constitutive luc activity in three quarters of the plants.

[b] *cir1* segregates as a monogenic recessive trait. In addition, the *PR-1a:luc* transgene segregates as a monogenic dominant trait and normally constitutive luc activity will only be visualised in three-quarters of the F2 plants containing the *cir1* mutation. The hypothesis was that *npr1*, which is insensitive to SA and segregates as a monogenic recessive trait, will block constitutive luc activity in one quarter of the F2 plants.

[c] *cir1* segregates as a monogenic recessive trait. In addition, the *PR-1a:luc* transgene segregates as a monogenic dominant trait and normally constitutive luc activity will only be visualised in three-quarters of the F2 plants containing the *cir1* mutation. In this case, the hypothesis was that *npr1* did not block constitutive luc activity in the F2 plants.

*F3 seedlings from both plants were tested for sensitivity to SA *in vitro*. Brown roots (indicative of the presence of *nahG*; Bowling et al. 1994) were not detected. Both F3 families were kanamycin resistant.

**All 11 F2 plants were tested for the presence of an NIa III restriction site in the wild type *NPR1* gene (Cao et al. 1997). The restriction site was lost in one of the 11 plants, indicating the presence of the *npr1-1* mutation (Clarke et al. 1998).

16 F2 plants scored, three contained the homozygous *npr1-1* mutation (results not shown). As the *cir1:npr1* double mutant expressed constitutive luc activity, it could be concluded that second hypothesis was correct, namely that *npr1* does not suppress constitutive *PR-1* activity in *cir1* plants.

The influence of SA application on constitutive PR-1 and PDF1.2 expression in cir1. *cir1* expresses *PR-1a:luc*, *PR-1* and *PDF1.2* constitutively. In order to investigate further the relationship between *cir1* and SA, *cir1* and *PR-1a:luc* Col-0 plants were inoculated with *Pst* DC3000 *(avrB)* or 1mM SA. Leaf samples were assayed for luc activity using the luminometer assay (Fig.5.4b) or subjected to Northern blot analysis using the *PR-1* and *PDF1.2* probes (Fig.5.4a). In the case of the *PR-1a:luc* Col-0 plants, addition of SA or inoculation with *Pst*DC3000 *(avrB)* induced both luc activity (Fig.5.4b) and *PR-1* expression (Fig.5.4a), which corresponded to previous results (Fig.3.2, Fig.4.1, Fig.4.2, Fig.4.3, Fig.4.4). Inoculation of Col-0 plants with *Pst*DC3000 *(avrB)* induced *PDF1.2* expression, whereas SA application had no effect on *PDF1.2* expression (Fig.5.4a). This result corresponds to previous reports (Penninclx et al. 1996, Malek & Dietrich 1999). In the case of *cir1*, addition of SA or inoculation with *Pst*DC3000 *(avrB)* induced both luc activity (Fig.5.4b) and *PR-1* expression (Fig.5.4a) to levels approximately 10-fold higher than those found in naïve *cir1* plants. This indicates that *cir1* is some how primed or 'potentiated' for SA-induction of *PR-1* expression. However, inoculation of *cir1* with *Pst*DC3000 *(avrB)* partially suppressed *PDF1.2* expression and SA application completely suppressed *PDF1.2* expression in comparison to naïve *cir1* plants (Fig.5.4a). Thus, applied SA in *cir1* plants appears to play a positive role in elevating *PR-1* expression, but plays a negative role by suppressing *PDF1.2* expression.

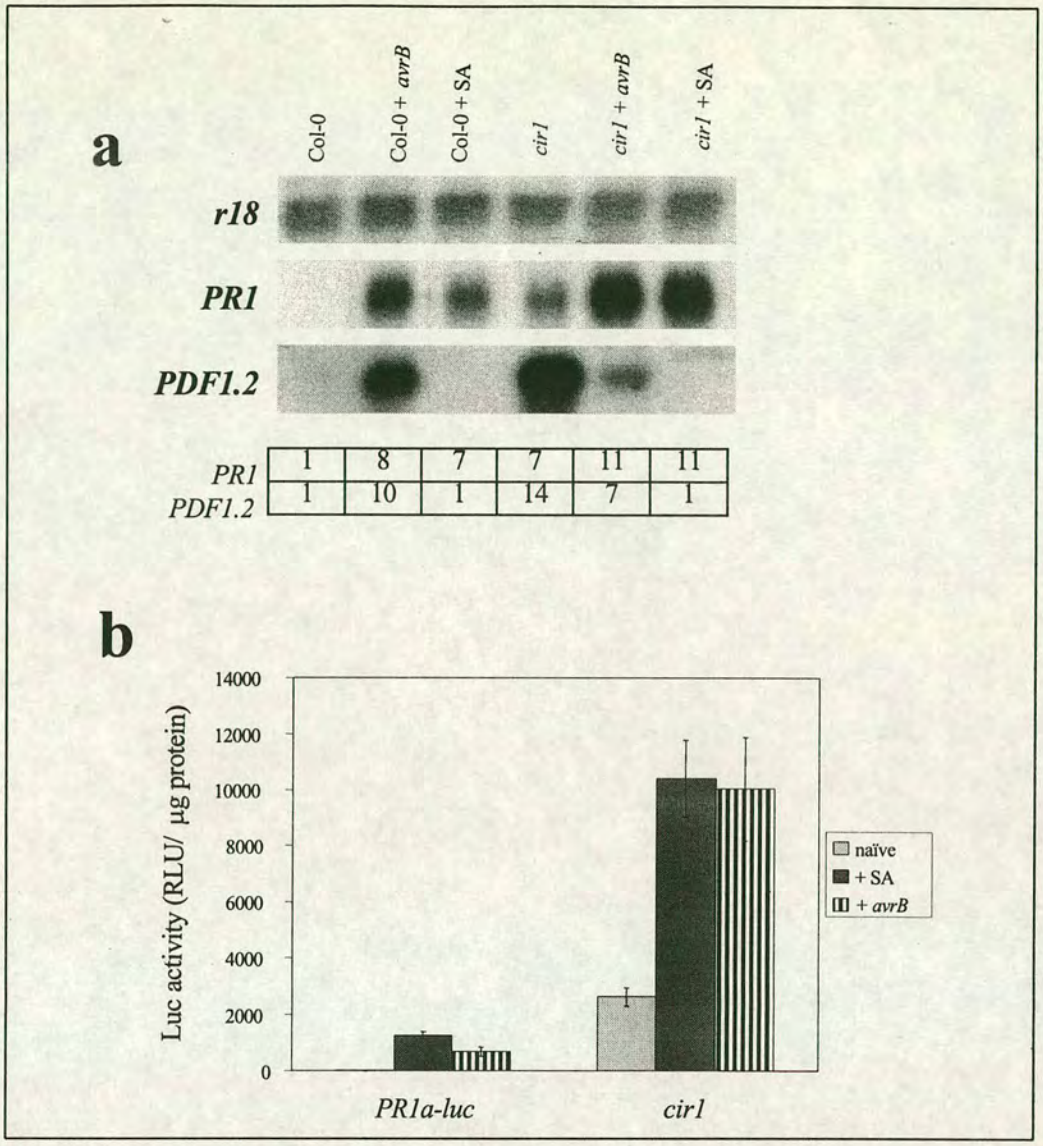

Fig.5.4. *PR-1* expression is elevated and *PDF1.2* expression is suppressed by SA application in *cir1*.

a: Northern blot analysis of *PR-1* and *PDF1.2* mRNA expression in Col-0 and *cir1*. Naïve plants, plants treated with 1mM SA and harvested after 24 hours, or plants inoculated with *Pst*DC3000 (*avrB)* and harvested after 48 hours were analysed. RNA samples were sequentially probed with the *PR-1, PDF1.2* and *r18* probes. The table below the blot represents the fold induction of the gene expression for each sample relative to Col-0. The expression was quantified using the ImageQuant software, adjusted to the expression of the loading control (*r18)* and normalised to the expression of Col-0, which was set to 1 unit. *avrB*, plants inoculated with *Pst*DC3000 (*avrB*).

b: Luminometer assay of *cir1* and *PR-1a:luc* plants.

Naïve plants, plants treated with 1mM SA and harvested after 24 hours, or plants inoculated with *Pst*DC3000 (*avrB)* and harvested after 36 hours were analysed for luc activity. Presented values are averages of readings from four plants. Error bars represent the standard error between values at the 95% confidence level.

5.5. Relationship of *cir1* to ethylene

Ethylene, in combination with jasmonates, has been shown to play an important role in signal transduction leading to *PDF1.2* expression (Penninckz et al. 1998).. As naïve *cir1* plants express strong constitutive *PDF1.2* expression, it was decided to investigate the level of ethylene evolution in *cir1* plants.

Ethylene readings using a gas chromatograph were conducted by Dr Pietro Ianetta, at the Scottish Crop Research Institute, Invergowrie, Dundee and the results obtained are outlined in Fig.5.5. As ethylene is released upon virulent pathogen infection (reviewed in Johnston & Ecker 1998), Col-0 plants inoculated with virulent *Pst*DC3000 were included as a positive control. These plants were assayed three days after inoculation. Ethylene evolution in wild type Col-0 plants was low (Fig.5.5) and corresponded to levels previously obtained for light-grown Arabidopsis plants (Guzman & Ecker 1990). However, ethylene evolution in *cir1* plants was significantly more than in Col-0 plants (Fig.5.5). A one-way ANOVA test was performed which showed a statisitically significant difference in ethylene evolution between Col-0 and *cir1*. Ethylene evolution by naïve *cir1* plants was equivalent to ethylene evolution by Col-0 plants inoculated with virulent *Pst*DC3000 (Fig.5.5).

When Arabidopsis seedlings are grown in the dark in the presence of ethylene, they under go the 'triple response', which consists of an inhibition of root and hypocotyl elongation, radial swelling of the hypocotyl and an exaggerated curvature of the apical hook (Ecker 1995). A number of Arabidopsis mutants have been isolated which display a constitutive triple response and greater ethylene evolution (Guzman & Ecker 1990, Kieber et al. 1993, Roman et al. 1995). In order to test *cir1* for a constitutive triple response *in vitro*, *cir1* seeds were plated out on to medium containing 10μM 1-amino-cyclopropane-1-carboxylic acid (ACC). ACC is the immediate precursor to ethylene in the biosynthetic pathway (Kende 1993) and its inclusion in tissue culture medium is a convenient alternative method for testing the ethylene triple response. After 5 days in the dark on MS medium supplemented with 10μM ACC, Arabidopsis seedlings have germinated and root inhibition in Col-0

wild-type is clearly seen (Alonso et al. 1999). ACC inhibited rooting in *cir1* seedlings and *cir1* seedlings did not show inhibition of rooting in the absence of ACC (results not shown). Thus, *cir1* responded as Col-0 wild-type to ACC and did not show constitutive expression of the triple response.

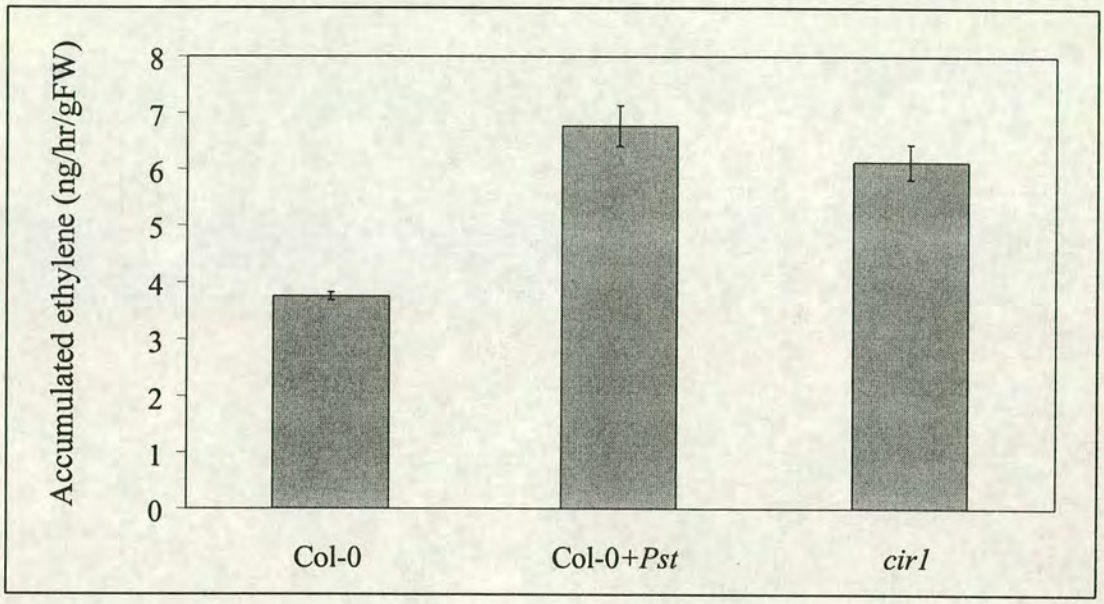

Fig.5.5. Comparison of ethylene evolution in Col-0, Col-0 inoculated with *Pst*DC3000 and *cir1*.

Five-week old plants were enclosed in a container and ethylene produced over a 24 hour period was collected. Ethylene concentration was determined by GC analysis. The values presented are the average of six replicates ± SE (nanograms ethylene produced per gram of fresh weight plant material).

5.6. Epistasis between *cir1, ein2.1* and *jar1*

Isolation of cir1:ein2.1 and cir1:jar1 double mutants

In order to determine the role of ethylene and jasmonates in constitutive *PDF1.2* expression and disease resistance in *cir1* plants, *cir1* was crossed with the ethylene insensitive mutant *ein2.1* (*e*thylene *in*sensitive *2.1*) (Guzman & Ecker 1990) and the Me-JA-insensitive mutant *jar1* (Staswick et al. 1992). As all three mutations (*cir1, ein2.1* and *jar1*) were recessive, double mutants from the *cir1*X*ein2.1* and *cir1*X*jar1* crosses were identified in the F2 populations from each cross (Table 5.6).

As *cir1* segregates as a monogenic recessive trait and the *PR-1a:luc* transgene segregates as a monogenic dominant trait, constitutive luc activity will normally only

be visualised in three-quarters of the F2 plants containing the *cir1* mutation. In the case of both the segregating F2 populations from the *cir1* X *ein2.1* cross and the *cir1* X *jar1* cross, the hypothesis was that the second mutation would have no influence on *cir1*-induced constitutive luc activity. Segregation data from both F2 populations confirmed these hypotheses (Table 5.6). F2 plants which produced luc constitutively were allowed to self pollinate and F3 plants were analysed for either the *ein2.1* or *jar1* phenotype. *ein2.1* seedlings are insensitive to 10 μM ACC and produce roots *in vitro* (Alonso et al. 1999). One F3 family from the *cir1 X ein2.1* cross was identified which was insensitive to ACC in all seedlings, thus indicating that it represented a *cir1:ein2.1* double mutant. This line was used in further experiments. *jar1* plants are small and dark green in comparison to Col-0 and *jar1* seedlings produce longer roots when exposed to 10 μM Me-JA *in vitro* than Col-0 seedlings (Staswick et al. 1992). One F2 plant from the *cir1* X *jar1* cross produced both luc constitutively and had a small dark green phenotype indicative of *jar1* (Table 5.6). F3 seedlings from the self-pollination of this F2 plant produced longer roots when exposed to 10 μM Me-JA *in vitro* than Col-0 or *cir1*, indicating that this line represented a *cir1:jar1* double mutant.

A cross was also made between the dominant ethylene-insensitive mutant *etr1* (Bleecker et al. 1988) and *cir1*. F1 plants from this cross were identified (Table 5.6) but due to time constraints, F2 plants were not analysed. Two attempts were also made to cross *cir1* with the Me-JA-insensitive mutant *coi1* (Feys et al. 1994). However, *coi1* plants became infected with powdery mildew in the greenhouse at both attempts and died before silliques had matured.

Table 5.6. Genetic analysis of *cir1* X *ein2.1* and *cir1* X *jar1*.

Generation	Donor	Recipient	Hypothesis	No. tested	Luc activity		No luc activity		χ^2	*P=5%*	Accept?
					Observed	Expected	Observed	Expected			
F1	*cir1*	*ein2.1*		6*	0	0	6	6			
F2	*cir1*	*ein2.1*	3:13[a]	22	5**	4	17	18	0.306	<3.841	Yes
F1	*cir1*	*etr1*		3*	0	0	3	3			
F1	*cir1*	*jar1*		5*	0	0	5	5			
F2	*cir1*	*jar1*	3:13[b]	68	17***	13	51	55	1.806	<3.841	Yes

The χ^2 value was calculated and compared to the critical value at the 5% probability level (Ennos 2000).

[a] *cir1* segregates as a monogenic recessive trait. In addition, the *PR-1a:luc* transgene segregates as a monogenic dominant trait and normally constitutive luc activity will only be visualised in three-quarters of the F2 plants containing the *cir1* mutation. In this case the hypothesis was that *ein2.1* will have no effect on luc activity.

[b] *cir1* segregates as a monogenic recessive trait. In addition, the *PR-1a:luc* transgene segregates as a monogenic dominant trait and normally constitutive luc activity will only be visualised in three-quarters of the F2 plants containing the *cir1* mutation. In this case, the hypothesis was that *jar1* did not block constitutive luc activity in the F2 plants.

* These F1 plants were identified as kanamycin resistant *in vitro*, thus indicating that the transformation cassette containing the *PR-1a:luc* gene and the kanamycin resistance gene had been donated by the *cir1* pollen donor.

**F3 seedlings from these plants were tested for sensitivity to ACC *in vitro*. When grown in the dark, Col-0 seedlings do not root on medium containing ACC, whereas *ein2.1* seedlings are insensitive to ACC. One line was insensitive to ACC, thereby defining a *cir1:ein2.1* double mutant.

***One of these F2 plants also showed the *jar1* phenotype (small, dark green leaves in comparison to Col-0). F3 seedlings derived from self-pollination of this F2 plant were insensitive to Me-JA inhibition of rooting *in vitro*, thus defining a *cir1:jar1* double mutant.

Expression of PR-1 and PDF1.2 in cir1:ein2.1 and cir1:jar1.

Five-week old *cir1:ein2.1* and *cir1:jar1* plants were assayed for constitutive luc activity, and *PR-1* and *PDF1.2* expression (Fig.5.6). Constitutive luc activity was determined using the ultra-low light imaging camera (Fig.5.6b&d) and the luminometer assay (Fig.5.6c&e). Both methods showed that luc activity was reduced in *cir1:ein2.1* and *cir1:jar1* in comparison to *cir1* plants, both in the number of leaves per plant expressing *PR-1a:luc* (Fig.5.6b&d) and in average luc activity (Fig.5.6c&e). Three leaves chosen at random from a *cir1, cir1:ein2.1* and *cir1:jar1* plant and imaged in the ultra-low light camera are shown in Fig.5.6. All three *cir1* leaves expressed *PR-1a:luc* constitutively in both cases (Fig.5.6b&d), whereas only one *cir1:ein2.1* leaf out of three showed luc activity (Fig.5.6b) and two *cir1:jar1* leaves showed luc activity (Fig.5.6d). A luminometer assay of naïve *cir1* and *cir1:ein2.1* F3 plants showed an average five-fold reduction in luc activity in *cir1:ein2.1* in comparison to *cir1* (Fig.5.6c). *PR-1a:luc* expression was reduced approximately ten-fold in *cir1:jar1* F3 plants in comparison to *cir1* (Fig.5.6e). Luc activity was virtually undetectable in naïve *PR-1a:luc* transgenic plants (Fig.5.6c&e). Both of these results indicate that *PR-1a:luc* is expressed constitutively in *cir1:ein2.1* and *cir1:jar1* plants, but to levels much lower than in *cir1*. Thus, EIN2 and JAR1 appear to be partial requirements for constitutive luc activity in *cir1* plants.

Leaves from five-week old naïve Col-0, *ein2.1*, *cir1* and F3 *cir1:ein2.1* were harvested for Northern blot analysis with the *PR-1* and *PDF1.2* probes. Plants from all four lines were also treated with 1mM SA or inoculated with *Pst*DC3000 *(avrB)* and harvested for RNA extraction. Results from the Northern blot analysis are shown in Fig.5.6a. Col-0 and *ein2.1* samples did not express *PR-1* but treatment with 1mM SA or inoculation with *Pst*DC3000 *(avrB)* induced *PR-1* expression. The naïve *cir1* sample expressed *PR-1* constitutively and treatment with 1mM SA or inoculation with *Pst*DC3000 *(avrB)* induced *PR-1* expression to a higher level. Constitutive *PR-1* expression was lost in the *cir1:ein2.1* double mutant (Fig.5.6a), indicating that EIN2 is an absolute requirement for constitutive *PR-1* expression in *cir1*. Treatment of *cir1:ein2.1* plants with 1mM SA or inoculation with *Pst*DC3000 *(avrB)* induced

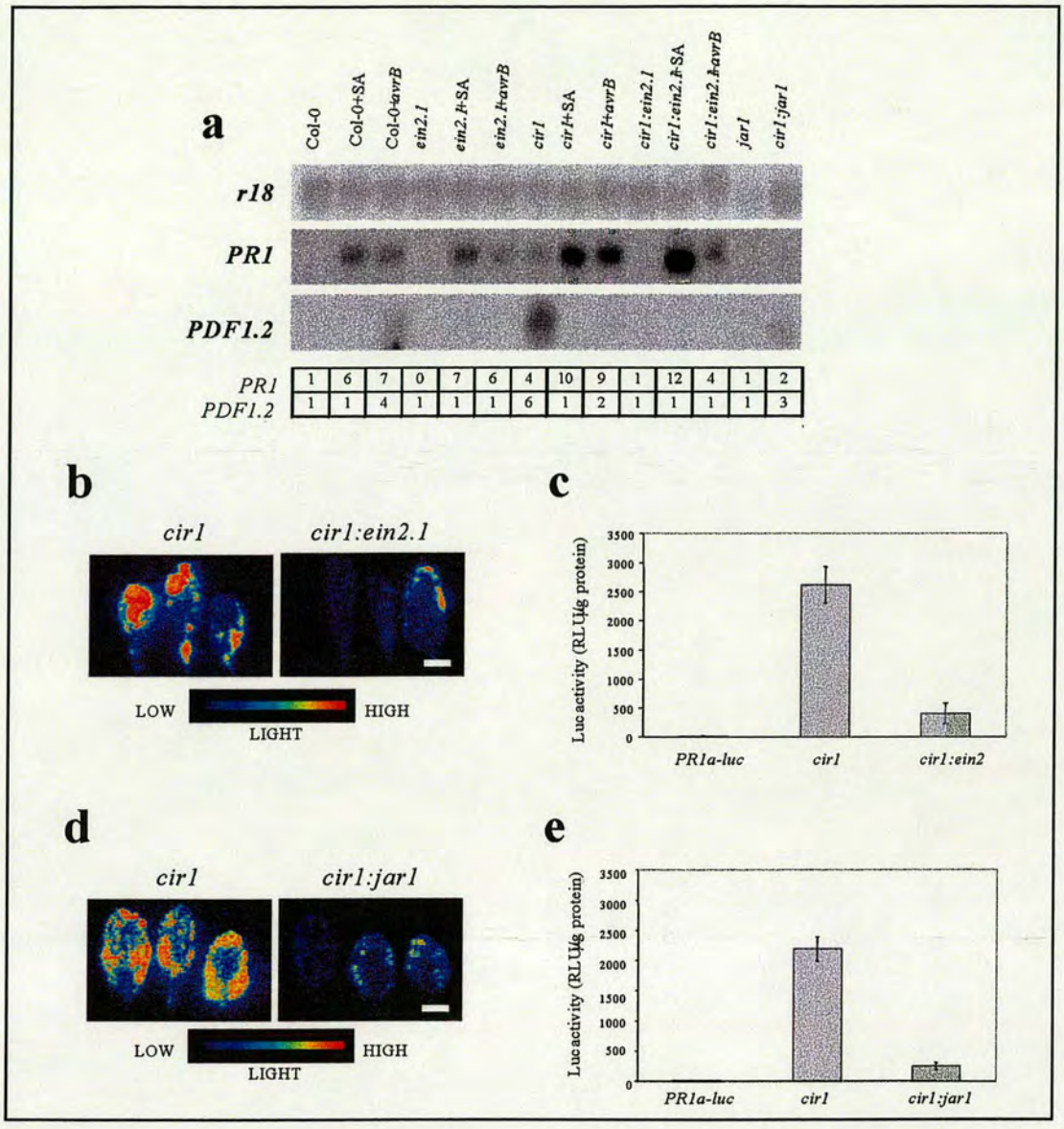

Fig.5.6. Expression of *PR-1* and *PDF1.2*, and production of luc activity in *cir1:ein2.1* and *cir:jar1* plants.

a: Northern blot analysis of *PR-1* and *PDF1.2* mRNA expression in Col-0, *cir1, ein2.1, jar1* and the *cir1:ein2.1* and *cir1:jar1* double mutants . Naïve plants, plants treated with 1mM SA and harvested after 24 hours, or plants inoculated with *Pst*DC3000 *(avrB)* and harvested after 48 hours were analysed. RNA samples were sequentially probed with the *PR-1, PDF1.2* and *r18* probes. The table below the blot represents the fold induction of the gene expression for each sample relative to Col-0. The expression was quantified using the ImageQuant software, adjusted to the expression of the loading control (*r18)* and normalised to the expression of Col-0, which was set to 1 unit. *avrB*, plants inoculated with *Pst*DC3000 (*avrB*).

b&d: Three representative leaves from five-week old *cir1* (b&d), *cir1:ein2.1* (b) and *cir1:jar1* plants (d) were painted with 1mM luciferin and imaged for constitutive *PR-1a:luc* production in the ultra low-light imaging camera.

c&e: Luminometer assay of *cir1 (*c&e), *PR-1a:luc* (c&e), *cir1:ein2.1* (c) and *cir1:jar1* (e) plants. Naïve plants were analysed for luc activity. Presented values are the averages of readings from four plants. Error bars represent the standard error between values at the 95% confidence level.

PR-1 expression (Fig.5.6a). *PDF1.2* was only expressed in the Col-0+*avrB* and naïve *cir1* samples (Fig.5.6a). Previously results indicated that *PDF1.2* was also weakly expressed in a *cir1+avrB* sample (Fig.5.4a) but this was not seen in this case. *PDF1.2* was not expressed in the *ein2.1+avrB* sample or in the naïve *cir1:ein2.1* sample (Fig.5.6a), indicating that EIN2 is an absolute requirement for *PDF1.2* expression in *cir1*. Furthermore, EIN2 also appears to be an absolute requirement for induction of *PDF1.2* expression in response to an avirulent bacterial pathogen.

In a preliminary experiment, leaves from a *jar1* plant and the F2 *cir1:jar1* plant were harvested for Northern blot analysis (Fig.5.6a). The *jar1* sample did not express either *PR-1* or *PDF1.2* (Fig.5.5a). Previous results (Fig.4.4a) indicated that *PR-1* and *PDF1.2* expression is induced in *jar1* by inoculation with *Pst*DC3000 *(avrB)*. Surprisingly, *PR-1* expression was lost in the *cir1:jar1* sample (Fig.5.6a). Weak *PDF1.2* expression was obtained in the *cir1:jar1* sample (Fig.5.6a), confirming that jasmonate signal transduction plays a partial role in *PDF1.2* expression in *cir1*.

Disease resistance of cir1:ein2.1 and cir1:jar1.

As both the *cir1:ein2.1* and *cir1:jar1* double mutants showed a huge reduction or complete loss of *PR-1* and *PDF1.2* expression, it was decided to test them for disease resistance. *cir1* plants showed enhanced resistance to both a virulent bacterial pathogen (*Pst*DC3000) and a virulent oomycete pathogen (*P.parasitica* Noco2). The aim of the disease resistance assays of the two double mutants was to determine the role of ethylene and jasmonate signal transduction in *cir1*-induced disease resistance.

The results of the disease resistance assays of *cir1:ein2.1* and *cir1:jar1* to *Pst*DC3000 and *P.parasitica* Noco2 are outlined in Fig.5.7. The positive control consisted of Col-0 plants pre-treated with 1mM SA twice, five and three days prior to pathogen inoculation. Either *npr1* or *nahG* plants were included as a negative control (results not shown). For the *Pst*DC3000 resistance assay, five-week old plants were inoculated with bacteria. After three days, inoculated leaves were harvested, ground and diluted in 10mM $MgCl_2$ buffer. Dilutions were plated out onto KB medium and the number of colony forming units (cfu) per leaf disk were

recorded. *Pst*DC3000 bacteria grew in Col-0 and *ein2.1* samples to similar levels while bacterial growth was reduced in *cir1* (Fig.5.7a). However, bacterial growth in *cir1:ein2.1* resembled that in *ein2.1*. A statistically significant difference in bacterial titre was found between *cir1* and both *ein2.1* and *cir1:ein2.1* using the Mann-Whitney test at the 95% confidence level (Minitab Version 12). These results indicate that *cir1*-induced resistance to *Pst*DC3000 is dependent on EIN2 and ethylene signal transduction. In the *Pst*DC3000 disease resistance assay for *cir1:jar1*, bacteria grew in Col-0, *jar1* and *cir1:jar1* plants to similar levels whereas bacterial growth was reduced in *cir1* (Fig.5.7c). A statistically significant difference in bacterial titre was found between *cir1* and both *jar1* and *cir1:jar1* using the Mann-Whitney test at the 95% confidence level (Minitab Version 12). This also indicates that *cir1*-induced resistance to *Pst*DC3000 is dependent on JAR1 and jasmonate signal transduction. Bacterial growth in *cir1* plants in both experiments was less or equivalent to bacterial growth in Col-0 plants treated with SA (Fig.5.7a&c), indicating that *cir1*-induced resistance to *Pst*DC3000 is equivalent to SA-induced resistance.

For the *P.parasitica* Noco2 resistance assay, four-week old plants were sprayed with a conidiospore solution. After incubation in a humid environment for 10 days, plants were scored for conidiophore production and a disease index was calculated (Fig.5.7b&d). The Col-0 and *ein2.1* samples showed equivalent disease development, while *cir1* and *cir1:ein2.1* showed reduced disease development (Fig.5.7b). Thus, *cir1*-induced resistance to *P.parasitica* Noco2 appears to be independent of EIN2 and ethylene signal transduction. In Fig.5.7d, Col-0 and *jar1* plants also showed equivalent disease development, whereas *cir1* and *cir1:jar1* showed a reduced disease index. Thus, *cir1*-induced resistance to *P.parasitica* Noco2 appears to be independent of JAR1 and jasmonate signal transduction. Furthermore, disease development on *cir1* plants was equivalent to disease development on Col-0 plants treated with SA (Fig.5.7b&d), indicating that *cir1*-induced resistance to *P.parasitica* Noco2 is equivalent to SA-induced resistance.

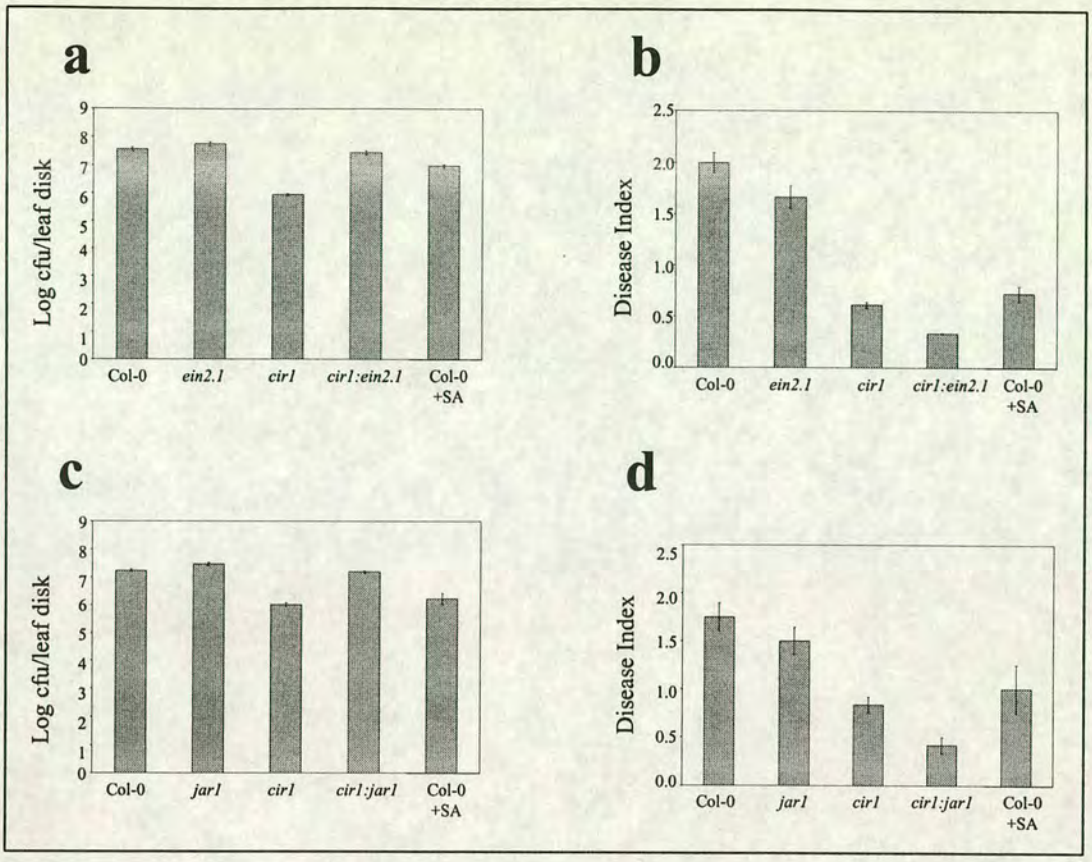

Fig.5.7. Disease resistance of *cir1:ein2.1* and *cir1:jar1*.
a&c: Resistance to *Pst*DC3000.
b&d: Resistance to *P.parasitica* Noco2.
a: Graph indicating bacterial titre three days after *Pst*DC3000 inoculation. Col-0, *ein2.1*, *cir1*, *cir1:ein2.1* and Col-0 plants treated with SA (Col-0+SA) were tested.
b: Disease rating of *P.parasitica* Noco2 infection 10 days after inoculation. Col-0, *ein2.1*, *cir1*, *cir1:ein2.1* and Col-0 plants treated with SA (Col-0+SA) were tested.
c: Graph indicating bacterial titre three days after *Pst*DC3000 inoculation. Col-0, *jar1*, *cir1*, *cir1:jar1* and Col-0 plants treated with SA (Col-0+SA) were tested.
d: Disease rating of *P.parasitica* Noco2 infection 10 days after inoculation. Col-0, *jar1*, *cir1*, *cir1:jar1* and Col-0 plants treated with SA (Col-0+SA) were tested.
Three plants per line were analysed in the *Pst*DC3000 assay and 12 plants per line were analysed in the *P.parasitica* Noco2 disease resistance assay. Presented values represent average values. Error bars represent the standard error between replicates at the 95% confidence level. Pathogen inoculation techniques and scoring methods are outlined in Chapter 2.

5.7. Discussion

The *cir1* mutant expressed *PR-1, PR-2, PR-5, PDF1.2,* and *GST1* constitutively and showed enhanced resistance to both a virulent bacterial pathogen and a virulent oomycete pathogen. Further, more detailed characterisation of *cir1* in terms of phenotype, genetic analysis, relationship to the signalling molecules SA and ethylene

and epistatic interactions with SA-, Me-JA- and ethylene-insensitive mutants was presented in this chapter.

cir1 plants do not show any dramatic changes in morphology in comparison to wild-type Col-0 (Fig.5.1b) and do not display macro- or micro-lesion development (Fig.5.2), but are slightly smaller in comparison to Col-0 (Table 5.1). However, *cir1* plants accumulated SA to levels approximately ten times higher than wild-type Col-0 (Fig.5.3). The *cpr6* mutant, which does not show spontaneous formation of macro- or micro-lesions (Clarke et al. 1998), and the *cep* mutant, which shows spontaneous lesions formation (Silva et al. 1999), are comparable to *cir1* in terms of SA accumulation. Levels of free SA in the *cpr6* and *cep* mutants were seven times higher than in naïve Col-0 plants (Clarke et al. 1998, Silva et al. 1999). However, *cpr1* and *cpr5* accumulate SA to much higher levels, approximately 30 times more than naïve Col-0 plants (Bowling et al. 1994, Bowling et al. 1997). SA accumulation in Arabidopsis is often associated with formation of lesions, either to high levels in local tissue following inoculation with an avirulent pathogen (Lawton et al.1995, Summermatter et al. 1995), to lower levels in systemic tissue during SAR (Lawton et al.1995, Summermatter et al. 1995) or through constitutive expression in mutants such as *cpr5* (Bowling et al. 1997), *cep* (Silva et al. 1999) and *acd2* (Greenberg et al. 1994). Arabidopsis mutants accumulating SA to high levels may also display an abnormal morphology. For example, the *cpr1* mutant shows a stunted morphology in comparison to Col-0 wild-type plants (Bowling et al. 1994) and *cpr6* mutants show loss of apical dominance in both *cpr6/CPR6* and *cpr6/cpr6* plants (Clarke et al. 1998). Thus, *cir1* appears to describe a novel class of constitutive SAR mutants, where SA accumulates to higher levels with out dramatically influencing plant morphology. It is possible that SA accumulation in *cir1* is below the threshold level needed to bring about a extreme change in morphology such as stunting, or that accumulated SA in *cir1* has a cellular localisation that does not greatly interfere with plant development. Furthermore, it is possible that a HR-independent signalling pathway leads to accumulation of SA in *cir1*, in a manner similar to that in the *dnd1* mutant (Yu et al.1998). Identification and characterisation of a *cir1:nahG* double mutant will help to determine the role of accumulated SA in *cir1*. Unfortunately, a

cir1:nahG F2 double mutant could not be isolated using the ultra low-light imaging camera as constitutive luc activity was dependent on SA accumulation. Cloning and sequencing of the *CIR1* gene should facilitate the production of a *CIR1* PCR marker. Identification of homozygous *cir1* plants with this marker in combination with the brown root phenotype of *nahG* plants on SA (Bowling et al.1994) could thus provide a method for identifying a *cir1:nahG* F2 double mutant.

Light-grown *cir1* plants also evolved ethylene to levels approximately twice higher than wild-type Col-0 (Fig.5.5). Ethylene evolution in *cir1* is comparable to the *eto1* (*et*hylene *o*verproducer *1*), *eto3* and *ein2.1* mutants (Guzman & Ecker 1990, Kieber et al. 1993), where ethylene is also produced in light-grown plants to levels approximately twice those of wild-type. Ethylene evolution was also determined in *acd5* mutant plants, which show spontaneous cell death, SA accumulation and expression of defence-related genes in older plants (Greenberg et al. 2000). Ethylene evolution in *acd5* plants showing lesions was approximately 5-fold more than wild-type, whereas younger *acd5* plants without lesions evolved approximately twice as much ethylene as wild-type plants (Greenberg et al. 2000).

Ethylene signal transduction through EIN2 is required for luc activity and *PR-1* expression in *cir1* (Fig.5.6). It is interesting to note that *PR-1a:luc* expression in *cir1:ein2.1* is reduced in comparison to *cir1* whereas *PR-1* expression is abolished in *cir1:ein2.1*. This may reflect different ethylene signal transduction requirements for transcription of the Arabidopsis *PR-1* gene in comparison to transcription of the *luc* reporter gene as controlled by the tobacco *PR-1a* promoter. Both the Arabidopsis *PR-1* (Lebel et al. 1998) and tobacco *PR-1a* (Payne et al. 1988) promoters contain the ethylene response *cis* element ERELEE4 isolated in tomato (Montgomery et al. 1993), indicating that ethylene plays a role in transcription of these promoters. A 903 bp promoter fragment was used to generate the *PR-1a:luc* transgenic plants. It is possible that ethylene signal transduction is also required for binding of transcription repressors to *cis* elements upstream of the 903 fragment, which are not present in the *PR-1a:luc* transgenic line. If this is occurring, it would help to explain different

levels of *PR-1* expression obtained for *cir1:ein2.1* in the luminometer and Northern blot analyses (Fig.5.6).

Results from a preliminary experiment with the *cir1:jar1* F2 plant suggested that jasmonate signal transduction through JAR1 is required for *PR-1* expression (Fig.5.6a)[a]. This result was suprising as induction of *PR-1* expression by an avirulent pathogen was previously shown to be independent of jasmonate signal transduction (Pieterse et al. 1998, Thomma et al. 1998). Furthermore, *PR-1* expression was induced in *jar1* plants by inoculation with *Pst*DC3000 (*avrB*) (Fig.4.4a). Interestingly, it has been reported that expression of the acidic *PR-1b* gene in tobacco is induced by either a combination of Me-JA and ethylene, or by a combination of Me-JA and SA (Xu et al. 1994). Thus, Me-JA could play a role in *PR-1* expression under certain conditions.

It is interesting to note that constitutive luc activity is maintained in the *cir1:npr1* double mutant (Table 5.5), indicating that *PR-1a:luc* expression is independent of NPR1 in *cir1*. Further experiments with the *cir1:npr1* double mutant should help to elucidate the function of NPR1 in *PR-1* expression and disease resistance in *cir1*.

Ethylene signal transduction through EIN2 and jasmonate signal transduction through JAR1 are required for *cir1*-induced constitutive *PDF1.2* expression (Fig.5.6a). Previously it was shown that concominant ethylene and jasmonate signal transduction were required for *PDF1.2* expression in Arabidopsis (Penninckx et al. 1998). More recently, it was shown that Me-JA-treated transgenic Arabidopsis plants expressing the carboxy-end of the EIN2 protein expressed *PDF1.2* to high levels (Alonso et al. 1999), indicating that EIN2 is the link between ethylene and jasmonate signal transduction leading to *PDF1.2* expression. Results obtained in Fig.5.6a, where *PDF1.2* expression is abolished in *cir1:ein2.1*, confirm this observation. Expression of *PDF1.2* in the *cir1:jar1* double mutant was partly

[a] The Northern blot of *cir1:ein2* and *cir1:jar1* with the *PR-1* probe was repeated after submission of the thesis. *PR-1* expression was seen in both double mutant lines, but at a reduced level in comparison to *cir1*. Thus, signal transduction through EIN2 and JAR1 does not appear to be an absolute requirement for *CIR1*-induced *PR-1* expression, but does play a partial role.

suppressed (Fig.5.6a), indicating that jasmonate-signalling through JAR1 is only partially required for *PDF1.2* expression in *cir1*.

Ethylene signal transduction through EIN2 and jasmonate signalling through JAR1 was required for *cir1*-induced enhanced resistance to *Pst*DC3000 but was not required for resistance to *P.parasitica* Noco2 (Fig.5.7). Previously it was shown that growth of avirulent and virulent *Pst*DC3000 in *ein2* was equivalent to Col-0, implying that ethylene signal transduction did not play a role in basal *Pst*DC3000 resistance (Bent et al. 1992). *Pst*DC3000 grew to similar levels in Col-0 and *ein2.1* (Fig.5.7a), confirming that ethylene signal transduction through EIN2 is not required for basal resistance to *Pst*DC3000. Bacterial growth in *cir1* plants was reduced but not in *cir1:ein2.1* plants (Fig.5.7a), indicating that ethylene signal transduction is required for *CIR1*-induced resistance to *Pst*DC3000. SAR to virulent *Pst*DC3000, as induced by inoculation with *Pst*DC3000 (*avrRpt2*), was also abolished in *ein2.1* in addition to other ethylene-insensitive mutants (Knoester et al. 1999). On the other hand, reduced *P.parasitca* Noco2 disease symptoms were observed for both *cir1* and *cir1:ein2.1* (Fig.5.7b). Previous reports have indicated that ethylene signal transduction through EIN2 is neither required for basal resistance (Thomma et al. 1998) nor SAR to *P.parasitica* induced by SA or *Pst*DC3000 *(avrRpt2)* (Lawton et al. 1994, Lawton et al. 1995). The results in Fig.5.7 confirm that ethylene signal transduction through EIN2 is not required for induced resistance to *P.parasitica* Noco2.

*Pst*DC3000 grew to similar levels in Col-0 and *jar1* (Fig.5.7c), indicating that jasmonate signal transduction is not required for basal resistance to *Pst*DC3000. Bacterial growth was reduced in *cir1* plants but not in *cir1:jar1* plants (Fig.5.7c), indicating that JA signal transduction through JAR1 plays a role in *cir1*-induced SAR against *Pst*DC3000. Previously it was shown that SAR to virulent *Pst*DC3000, as induced by inoculation with *Pst*DC3000 (*avrRpt2*), was not abolished in *jar1* and thus that SAR was independent of jasmonate signal transduction (Pieterse et al. 1998). Thus, results presented in Fig.5.7c for the *cir1:jar1* mutant further suggest that *cir1* may define a novel jasmonate-dependent branch in the SAR signal

transduction network. Alternatively, accumulation of signalling molecules upstream of JAR1 in the SAR signal transduction network may negatively regulate *cir1*-induced *Pst*DC3000 resistance through a cross-talk mechanism.

Reduced *P.parasitica* Noco2 disease symptoms were observed for both *cir1* and *cir1:jar1* in comparison to Col-0 and *jar1* (Fig.5.7d). It has previously been reported that jasmonate signal transduction through COI1 is not required for resistance to *P.parasitica* (Thomma et al. 1998). Thus, the results in Fig.5.7d confirm that jasmonate signal transduction is not required for induced resistance to *P.parasitica* Noco2.

When *cir1* plants were treated with SA or inoculated with *Pst*DC3000 *(avrB)*, *PR-1a:luc* expression in a luminometer assay and *PR-1* expression in Northern blot analysis were substantially increased in comparison to naïve *cir1* plants (Fig.5.4). This indicates that flux through the SA-dependent pathway is not completely 'on' in *cir1* and *PR-1* expression can be boosted by the addition of SA. This response is comparable to *cpr1* and *cpr6*. When *cpr1* was treated with SA or INA, *PR-2:GUS* activity and *PR-1* expression was increased relative to naïve *cpr1* plants (Bowling et al. 1994). *PR-1* expression was slightly increased in the *cpr6* + INA sample relative to naïve *cpr6* plants (Clarke et al. 1998). However, addition of SA completely inhibited constitutive expression of *PDF1.2* in *cir1* (Fig.5.4), which is also analogous to *PDF1.2* expression in *cpr6* plants treated with INA (Clarke et al. 1998). *PDF1.2* expression was also substantially reduced in *cir1* plants inoculated with *Pst*DC3000 *(avrB)* whereas *PDF1.2* expression in Col-0 was induced by avirulent *Pst* (Fig.5.4, Malek & Dietrich 1999). This indicates that the signal transduction pathway leading to *PDF1.2* expression in *cir1* is completely 'on' and that extra flux through this pathway or flux through a cross-talk pathway results in reduction in *PDF1.2* expression. Previously, it was shown that SA prevented wound-induced gene expression in tomato by blocking synthesis of JA (Pena-Cortes et al. 1993). As SA is known to accumulate following inoculation with both avirulent and virulent *Pst* (Cameron et al. 1999), it is possible that accumulation of SA over a threshold level in *cir1* may be blocking synthesis of JA, thereby blocking *PDF1.2* expression. Accordingly, SA

accumulation in Col-0 inoculated with *Pst*DC3000 may fall below the threshold level, thereby not influencing the expression of *PDF1.2* by 'cross-talk'.

Based on genetic analysis, the *cir1* trait is inherited as a recessive mutation at a single locus (Table 5.2). Furthermore, it was found that the *ceb3* mutation was allelic to *cir1* in complementation analyses (Table 5.3). The *cir1* mutation was mapped to the lower arm of chromosome 4, between the markers nga1111 and g4539 (Table 5.4). Unfortunately, no further PCR-based mapping markers were available between nga1111 and g4539 at the time of this study. However, a number of restriction fragment length polymorphisms (RFLPs), single nucleotide polymorphisms (SNPs) and visible markers are present in this 28cM region of chromosome 4 (http://www.arabidopsis.org). Utilisation of these markers and larger F2 mapping population from the *cir1*X L*er* cross should elucidate the fine map position of *cir1* (Lukowitz et al. 2000). Map-based cloning of *cir1* and sequencing of the isolated *CIR1* gene from wild-type Col-0, *cir1* and *ceb3* plants will show the position of the mutations in these alleles providing possible clues to *cir1* gene function.

In addition to *cir1*, four other recessive mutants (*cpr1, lsd1, acd2* and *cpr20*), which cause constitutive *PR* gene expression, elevated SA levels and enhanced disease resistance, have been mapped to chromosome 4 (Bowling et al. 1997, Dietrich et al. 1997, Greenberg et al. 1994, Silva et al. 1999). Of these, *cpr1* mapped to approximately the same region as *cir1* (Bowling et al. 1997, Table 5.4). The other three mutants mapped to positions on chromosome 4 distal to *cir1* so it was decided not to perform complementation analysis (Dietrich et al. 1997, Greenberg et al. 1994, Silva et al. 1999). To determine whether the mutation carried by *cpr1* was allelic to *cir1*, a cross was made between these two mutants and the resulting F1 plants were analysed for constitutive *PR-1a:luc* expression (Table 5.3). An unexpected segregation was obtained, as one of the 12 F1 plants analysed showed constitutive luc activity (Table 5.3). However, it is unlikely that *cir1* and *cpr1* are allelic. *cpr1* plants are stunted (Bowling et al. 1994) whereas *cir1* plants are only slightly smaller than Col-0. None of the F1 plants from the *cir1 X cpr1* cross were stunted. In

addition, *cpr1* plants do not express *PDF1.2* constitutively (Penninckx et al. 1996), whereas *cir1* plants do. Complementation analysis indicated that *cir1* and *cpr5*, a recessive mutant which also expresses constitutive SAR (Bowling et al. 1997), are not allelic (Table 5.3). This is consistent with mapping studies that have placed *cpr5* on chromosome 5 (Bowling et al. 1997).

Two dominant mutations (*acd6* and *ssi1*) also showed constitutive *PR* gene expression, elevated SA levels and enhanced disease resistance, and mapped to chromosome 4 (Rate et al. 1999, Shah et al. 1999). It was decided not to perform complementation analysis between these mutants and *cir1* as complementation between a dominant and a recessive mutation is difficult. It has been shown that *acd6* and *ssi1* are not allelic (Greenberg 2000). However, there is a small chance that *cir1* is allelic to either *acd6* or *ssi1* as they map to approximately the same 28cM region (Table 5.4, Rate et al. 1999, Shah et al. 1999). If this is the case, *cir1* and *ssi1* or *acd6* would identify different types of mutations in the same gene. The fine mapping of *ACD6, SSI1* and *CIR1* should determine whether this is the case or not.

Interestingly, *cir1* maps to a position close to Major Recognition Complex H (MRC-H), one of the many clusters of disease resistance genes that have been documented in Arabidopsis (Holub & Benyon 1997). Among others, MRC-H contains the *RPP5* resistance gene, which confers resistance to *P. parasitica* Noco2 in the L*er* ecotype only (Parker et al. 1993). Four ESTs showing similarity to resistance genes (R-ESTs) have been mapped within or close to MRC-H (Botella et al. 1997). Furthermore, the sequencing of chromosome 4 and analysis of the predicted proteins has indicated a function for more than 170 genes in disease resistance and almost 200 in signal transduction (Mayer et al. 1999). Thus, the proximity of *cir1* to both MRC-H and six enhanced disease resistance mutants on a chromosome rich in potential disease resistance genes is intriguing.

Chapter Six

General Discussion

SAR is triggered upon infection by a necrotising avirulent pathogen and is implicated in the subsequent development of resistance to a broad range of virulent plant pathogens (reviewed in Ryals et al. 1996). Elucidation of the mechanisms underlying SAR could contribute to the exploitation of SAR in controlling plant diseases. In the work described in this thesis, a transgenic Arabidopsis line was developed expressing the luciferase reporter gene under the control of the *PR-1a* promoter. This line was used to study SAR and a number of candidate SAR mutants were isolated using this line. Identification and characterisation of one mutant, *cir1*, which displayed constitutive activation of SAR, has provided new insights into the signal transduction network underlying SAR.

6.1. Luciferase activity accurately reports *PR-1* expression in *PR-1a:luc* plants

A homozygous *PR-1a:luc* transgenic Arabidopsis line was generated and in Chapter3, expression of *PR-1a:luc* was investigated. Luc activity was visualised by means of an ultra-low light imaging camera and a luminometer assay. Inoculation of *PR-1a:luc* plants with well-characterised inducers of SAR (avirulent bacterial pathogens) or application of SA, induced luc activity (Fig.3.2, Fig.3.3, Fig.3.4, Fig.5.4b). In Northern blot analysis, *PR-1* expression was also induced by application of SA or inoculation with the avirulent bacterial pathogen *Pst*DC3000 *(avrB)* (Fig.4.3, Fig.4.4a, Fig.5.4a, Fig.5.6a). Furthermore, luc activity and *PR-1* expression was induced congruently in *PR-1a:luc* plants following inoculation with *Pst*DC3000 (*avrB)* (Fig.3.3, Fig.3.4). Thus, luc activity accurately reports *PR-1* expression in *PR-1a:luc* plants.

In Chapter 3, the mutagenesis of *PR-1a:luc* seed and the mutant screen of M2 and M3 progeny was described. A number of candidate mutants, falling into different classes of abnormal luc activity, were uncovered. A sub-group of candidate mutants were carefully evaluated for abnormal luc activity during SAR by investigating expression of defence-related genes (Fig.4.3, Fig.4.4) and disease resistance (Fig.4.5,

Fig.4.6, Fig.4.7). Candidate mutants were only considered as bona fide SAR mutants if luc activity, defence-gene expression and disease resistance results correlated.

6.2. *cir1*: A novel SAR mutant

The *cir1* mutant was identified in Chapter 4. This mutant displayed constitutive expression of *PR-1a:luc* and defence-related genes (Fig.4.2, Fig.4.4). *cir1* also displayed constitutive activation of resistance to *Pst*DC3000, a virulent bacterial pathogen (Fig.4.5c), and *P.parasitica* Noco2, a virulent oomycete pathogen (Fig.4.6c). This analysis indicated that *cir1* is a bona fide SAR mutant. *cir1* defines a monogenic recessive mutation (Table 5.2) and maps to the lower arm on chromosome 4 (Table 5.4). Complementation analysis indicated that *cir1* is allelic to *ceb3*, an additional SAR mutant isolated in this study. Results obtained in Northern blot analyses and disease resistance assays for both *cir1* and *ceb3* are summarised in Table 6.1. Overall, *cir1* and *ceb3* display a similar phenotype, but with subtle differences. Both *cir1* and *ceb3* do not display spontaneous formation of HR-like lesions (Table 6.1). *ceb3* displayed stronger expression of the SA-dependent genes in comparison to *cir1* but *cir1* expressed *PDF1.2* more strongly than *ceb3* (Fig.4.4, Table 6.1). *cir1* was more resistant to *Pst*DC3000 than *ceb3* (Fig.4.5, Table 6.1) but *ceb3* was more resistant to *P.parasitica* Noco2 and *F.oxysporum* f.sp. *matthiolae* than *cir1* (Fig.4.6, Fig.4.7, Table 6.1). Although SA accumulation and ethylene evolution levels in *ceb3* were not determined, it is likely that production of both signalling molecules will be increased in comparison to wild-type Col-0, as for *cir1* (Table 6.1). Map-based cloning of *CIR1* and sequencing of the isolated *CIR1* gene from *cir1, ceb3* and wild-type Col-0 will provide comparison between different alleles and it will be interesting to determine the location of the mutations that convey these small differences in phenotype.

Table 6.1. Summary of results for *cir1* and *ceb3*

SAR feature	*cir1*	*ceb3*
Signalling molecule[a]		
ROS	no macro- or micro-lesions	no macro- or micro-lesions
SA	++	ND[b]
ethylene	+++	ND
Me-JA	ND	ND
Gene expression[a]		
PR-1	+	++
PR-2	+	++
PR-5	++	+++
GST1	+	+
Thi2.1	-	-
Lox2	-	-
PDF1.2	+++	++
ACS2	- (basal expression)	- (basal expression)
Disease susceptibility[c]		
*Pst*DC3000	+	++
P.parasitica Noco2	++	+
F. oxysporum f.sp. *matthiolae*	+++	++

[a]Denotes production of signalling molecules or expression of defence-related genes. -, no induction; + to +++ denotes extent of expression, with ++ the equivalent as Col-0 inoculated with *Pst*DC3000 (*avrB*).

[b]ND, not determined

[c]Denotes approximate disease susceptibility in comparison to Col-0. Susceptibility in Col-0 was set to +++ for all three disease resistance assays.

6.3. A model for *CIR1*-induced SAR signal transduction

Characterisation of *cir1* plants by analysing the accumulation of molecules involved in SAR signal transduction, the expression of defence-related genes, resistance to pathogens and epistatic interactions with other SAR signal transduction mutants has enabled the development of a model outlining the role of *CIR1* in SAR. This proposed model, which will evolve as more information about the function of the *CIR1* gene product becomes available, is outlined in Fig.6.1.

cir1 defines a recessive mutation in a single gene (Table 5.2). In a single step in a signal transduction pathway, production of a constitutive phenotype in the absence of any stimulus indicates the loss of a negative regulator (Bowler & Chua 1994). Thus, *CIR1* probably encodes a negative regulator of SAR (Fig.6.1). Signal components of SAR include ion fluxes, signal transduction via protein phosphorylation by activation of mitogen-activated protein kinase (MAPK) pathways and activation of transcription factors (Meskiene & Hirt 2000, Martin 1999, Yang et al. 1997). It is likely that *CIR1* encodes a component contributing to one of these processes. Map-based cloning of *CIR1* and analysis of the predicted protein structure should indicate the likely function of the CIR1 protein.

cir1 plants did not show spontaneous formation of HR-like macro-lesions (Fig.5.1b) or micro-lesions (Fig.5.2). Previous characterisation of spontaneous cell death in mutants displaying constitutive SAR has placed mutations either up-stream or down-stream of the HR in the SAR signal transduction network. Mutants displaying lesions such as *acd2, cpr5* and *cep* (Greenberg et al. 1994, Bowling et al. 1997, Silva et al. 1999), were placed upstream of the HR (Fig. 6.1). Mutants such as *cpr1* (Bowling et al. 1994) and *cpr6* (Clarke et al. 1998) which do not show spontaneous lesion formation were placed downstream of the HR (Fig. 6.1). However, recent isolation of the *dnd1* mutant, which displays constitutive expression of SAR but no HR development in response to inoculation with an avirulent bacterial pathogen (Yu et al. 1998), has brought the central role of the HR in the development of SAR into question. The *DND1* gene encodes a cyclic nucleotide-gated ion channel that

probably allows passage of Ca^{2+}, K^{+} and other cations (Clough et al. 2000). Transmembrane ion fluxes are one of the earliest detectable signalling events in plant defence responses (Hammond-Kosack & Jones 1996). Thus, *DND1* is likely to act very early in the induction of SAR. However, as *cir1* plants do not show spontaneous lesion development and a normal HR developed in *cir1* plants inoculated with *Pst*DC3000 *(avrB)* (results not shown), it is likely that *CIR1* acts downstream of the HR (Fig.6.1). Furthermore, both SA (Fig.5.3) and ethylene (Fig.5.5) were found to accumulate to higher levels in *cir1* plants in comparison to

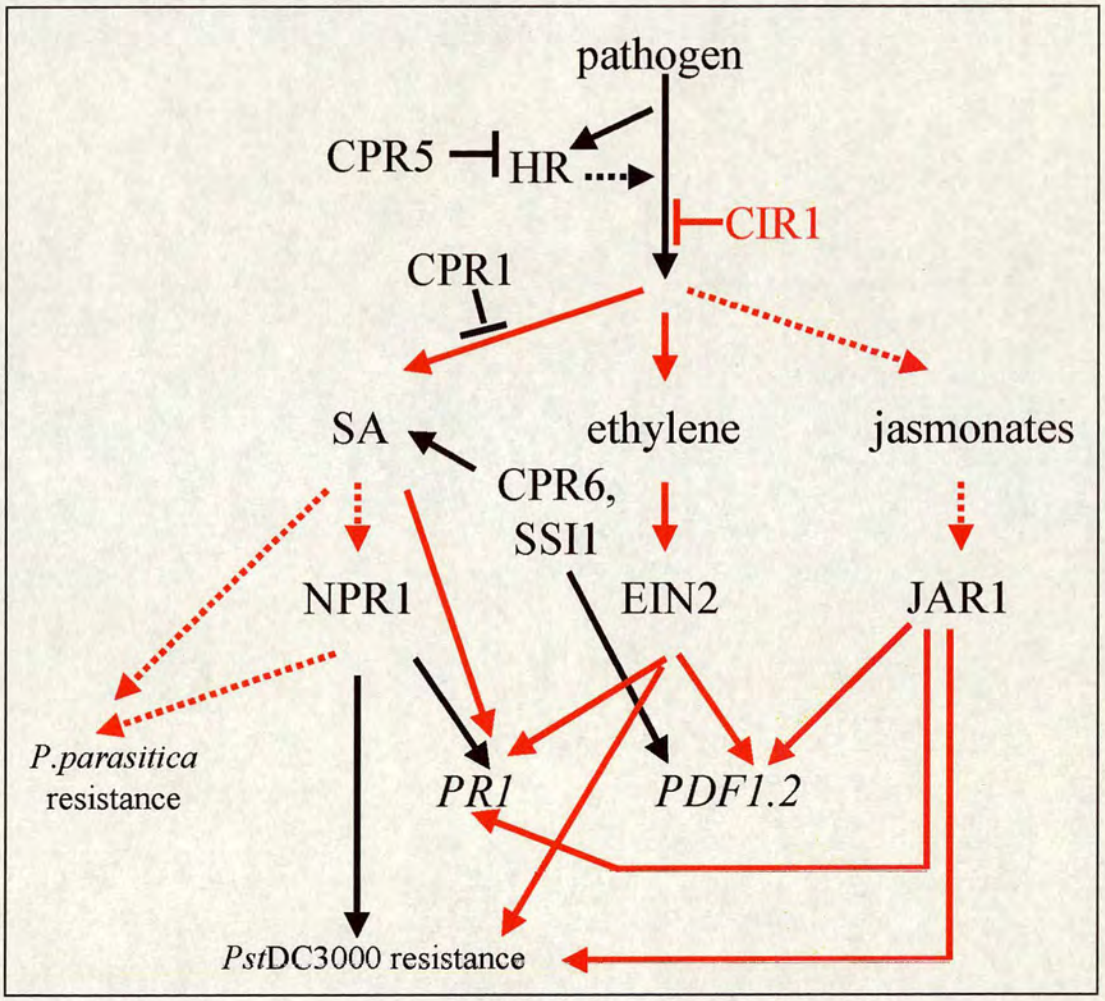

Fig.6.1. Proposed model of *CIR1*-induced SAR signal transduction.

Black lines indicate previous models (Shah et al. 1999, Clarke et al. 1998, Bowling et al. 1997, Bowling et al. 1994, Cao et al. 1994). Red lines indicate *CIR-1* induced SAR signal transduction. Dashed lines indicate putative signal transduction pathways. Arrows indicates positive action; blunt-ended lines indicate negative regulation.

wild-type plants, implying that *CIR1* acts upstream of the production of these signal transduction molecules in the SAR signal transduction network (Fig.6.1).

In addition to increased SA accumulation, *cir1* plants showed constitutive expression of the SA-dependent genes *PR-1, PR-2* and *PR-5* (Fig.4.4a&b), further indicating that *CIR1* plays an important role in SA-dependent SAR signal transduction. *cir1* plants also displayed constitutive expression of *PR-1a:luc* (Fig.4.2, Fig.5.1a) and luc activity reported the constitutive expression of *PR-1*. Analysis of the F2 progeny from a *cir1* X *nahG* cross (Table 5.5) indicated that constitutive luc activity (and thus *PR-1* expression) was dependent on SA accumulation (Fig.6.1). As the visual phenotype of *cir1* was dependent on the accumulation of SA, it was not possible to isolate a *cir1:nahG* double mutant for further analysis. However, resistance to virulent *Pst*DC3000 and *P.parasitica* Noco2 is associated with SA accumulation (Lawton et al. 1995), and thus it is likely that a *cir1:nahG* double mutant will be susceptible to these two pathogens. On the other hand, analysis of the F2 progeny from a *cir1* X *npr1* cross (Table 5.5) indicated that *PR-1* expression was independent of NPR1 (Fig.6.1). Expression of *PR-1* in the dominant mutants *cpr6* and *ssi1* was also SA-dependent but NPR1-independent (Clarke et al. 1998, Shah et al. 1999). Two signalling pathways, one dependent on SA and NPR1, and the other dependent on SA only, have thus been proposed to control the expression of *PR-1* during SAR (Shah et al. 1999, Clarke et al. 1998). Characterisation of the *sni1* mutant also suggested the presence of a second SA-dependent, NPR1-independet pathway (Li et al. 1999). A model was proposed whereby SNI1 represses the expression of *PR-1* in the absence of SA (Li et al. 1999, Delaney 2000). When SA is introduced, it activates NPR1, which represses the SNI1 repressor, allowing transcription of *PR-1*. Another SA-dependent, but NPR1 and SNI1-independent factor was identified that could also allow *PR-1* transcription (Li et al. 1999). Possibly this factor, *CPR6, SSI1* and *CIR1* are all involved in a second SA-dependent, NPR1-independent pathway leading to *PR-1* expression (Fig.6.1).

PR-1 expression in response to inoculation with an avirulent bacterial pathogen is neither dependent on *EIN2* (Fig.5.6a, Lawton et al. 1995) nor *JAR1* (Fig.4.3,

Fig.4.4a, Pieterse et al. 1998). However, *PR-1* expression in *cir1* was found to be dependent on *EIN2* and *JAR1* (Fig.5.6a). This implies that ethylene and jasmonates, in addition to SA, are required for signal transduction leading to *PR-1* expression in *cir1* (Fig.6.1). It is thus likely that the CIR1 protein acts as a negative regulator in a SA-, ethylene- and jasmonate-dependent pathway leading to *PR-1* expression, and further implies that *CIR1* must be operating at an early point in the SAR signal transduction network (Fig.6.1). The tunable dial model for the regulation of defence gene expression by SA, ethylene and jasmonates proposes that a plant is able to fine-tune it's response by employing a single signal molecule or a combination of the three molecules, depending on the nature of the attacking pathogen (Reymond & Farmer 1998). CIR1 appears to fit this model. Wild-type CIR1 is a negative regulator of SAR, presumably suppressing the expression of SAR genes in healthy tissue. Following pathogen attack, the plant 'tunes' the establishment of SAR by suppressing CIR1 activity, leading to the employment of all three signalling molecules in inducing *PR-1* expression. However, the pathogen that would suppress wild-type *CIR1* activity has yet to be identified, as it appears that *CIR1*-induced *PR-1* expression differs to *PR-1* expression induced by inoculation with *Pst*DC3000 *(avrB)*.

Expression of *PDF1.2* in *cir1* was dependent on *EIN2* and *JAR1* (Fig.5.6a), placing *PDF1.2* expression downstream of ethylene signalling through EIN2 and jasmonate signalling through JAR1 (Fig.6.1). It has previously been shown that *A.brassicicola*-induced *PDF1.2* expression occurs independently of SA (Penninckx et al. 1996) and that concominant jasmonate and ethylene signal transduction through EIN2 is required for *PDF1.2* expression (Penninckx et al. 1998, Alonso et al. 1999). Ethylene evolution in *cir1* plants was increased in comparison to wild-type Col-0 (Fig.5.5), further indicating that *PDF1.2* expression in *cir1* is dependent on ethylene signal transduction through EIN2 (Fig.6.1). Although jasmonate levels in *cir1* were not investigated in this study, Northern blot analysis indicates that it is unlikely that jasmonate production is increased in *cir1*. Treatment of *cir1* plants with SA suppressed *PDF1.2* expression (Fig.5.4a), indicating that increased SA accumulation in *cir1* plants may be suppressing jasmonate levels by a cross-talk mechanism.

Expression of the Me-JA responsive genes *Thi2.1* (Bohlmann et al. 1998) and *AtLOX2* (Bell & Mullet 1993) were also not detected in *cir1* (Fig.4.4c&d). It is possible that increased Me-JA levels *in planta* are required for *Thi2.1* and *AtLOX2* expression, but that endogenous jasmonate levels are sufficient, when ethylene signal transduction through EIN2 is enhanced, for expression of *PDF1.2*.

Enhanced resistance to the virulent bacterial pathogen *Pst*DC3000 in *cir1* is dependent on EIN2 and JAR1 (Fig.5.7a&c), placing *Pst*DC3000 resistance downstream of EIN2 and JAR1 in the model (Fig.6.1). Resistance to *Pst*DC3000 has previously been shown to be dependent on SAR signal transduction through SA and NPR1 (Cao et al. 1994, Bowling et al. 1994, Bowling et al. 1997, Clarke et al. 1998). Although *Pst*DC3000 resistance in *cir1* appears to be associated with *PR-1* and *PDF1.2* expression, characterisation of the *cpr6* mutant indicated that *Pst*DC3000 resistance could be uncoupled from *PR-1* expression, implying that the expression of additional NPR1-dependent genes are required for *Pst*DC3000 resistance (Clarke et al. 1998). It is likely that CIR1 also induces additional ethylene- and jasmonate-responsive genes that are required for *Pst*DC3000 resistance.

Enhanced resistance to the virulent oomycete pathogen *P.parasitica* Noco2 in *cir1* is independent of EIN2 and JAR1 (Fig.5.7b&d). Previously both NPR1-dependent and NPR1-independent *P.parasitica* resistance signalling has been reported (Cao et al. 1994, Bowling et al. 1997), although *P.parasitica* resistance is dependent on SA signalling (Lawton et al. 1994). It is thus likely that *CIR1*-induced resistance to *P.parasitica* Noco2 is dependent on SA signalling, either by an NPR1-dependent or an NPR1-independent signalling pathway (Fig.6.1). It has previously been proposed that NPR1-independent resistance to *P.parasitica* Noco2 is dependent on expression of *PDF1.2* (Bowling et al. 1997). Studies with the *cir1:ein2.1* and *cir1:jar1* double mutants in this thesis have shown that *PDF1.2* expression is suppressed without loss of *P.parasitica* Noco2 resistance, indicating that *P.parasitica* resistance is independent of *PDF1.2* expression (Fig.6.1).

6.4. Future analysis of the *CIR1*-induced SAR model

The proposal of a model outlining the role of *CIR1* in the SAR signal transduction network raises a number of additional questions. These are listed below. Due to time limitations, they could not be addressed in this thesis. Further characterisation of *cir1*, with the aim of answering these questions, should prove to be interesting and will help the development of the model presented in Fig.6.1.

- Expression of *PR-1* in *cir1* was dependent on SA but independent of NPR1. Is *CIR1*-induced resistance to *Pst*DC3000 and *P.parasitica* Noco2 dependent on NPR1? Disease resistance assays of the *cir1:npr1* double mutant should answer this question. Further Northern blot analysis should also uncover the role of SA and NPR1 in *CIR1*-induced constitutive *PDF1.2* expression.
- Ethylene evolution in *cir1* was increased in comparison to wild-type Col-0 plants and *CIR1*-induced ethylene signalling through EIN2 appears to play an important role in expression of *PR-1*and *PDF1.2*, and resistance to *Pst*DC3000. Is increased ethylene evolution in *cir1* due to increased biosynthesis of ethylene, or is it due to a feedback loop in ethylene signal transduction? Does CIR1 operate upstream or downstream of ethylene biosynthesis? Determination of *PR-1* and *PDF1.2* expression, and *Pst*DC3000 resistance in *cir1* plants treated with inhibitors of ethylene biosynthesis should help to answer these questions. Furthermore, characterisation of a *cir1:etr1* double mutant (which would contain a mutant ethylene receptor) would prove to be interesting.
- *CIR1*-induced *Pst*DC3000 resistance and expression of *PR-1* and *PDF1.2* appears to be dependent on jasmonate-dependent signalling through JAR1. What role are jasmonates playing in *cir1*? Is the production of jasmonates increased in *cir1*, or are endogenous levels sufficient for *CIR1*-induced signal transduction? Does CIR1 operate upstream or downstream of jasmonate production? Determination of the endogenous jasmonate levels (both JA and Me-JA) in *cir1* and wild-type Col-0 by GC-MS (Penninckx et al. 1996) should help to answer some of these questions. In addition, crossing *cir1* with the other Me-JA insensitive mutant *coi1* (Feys et al. 1994) and the JA-deficient triple mutant *fad3-*

2 fad 7-2 fad8 (McConn & Browse 1996), should indicate if *CIR1*-induced signalling is dependent on jasmonate signal transduction through JAR1 or on JAR1 only.

- *cir1* plants did not show spontaneous formation of lesions and showed enhanced resistance to *Pst*DC3000 and *P.parastica* Noco2 but not to *F.oxysporum* f.sp. *matthiolae.* Recently it has been shown that the *dnd1* mutant, which shows enhanced disease resistance without cell death, was resistant to *Botrytis cinerea* (Govrin & Levine 2000). Is *cir1* more resistant (or more susceptible) to other agriculturally important pathogens? Further disease resistance assays with additional pathogens such as *B. cinerea* should indicate if this is the case or not and further expand the role of CIR1 in SAR.
- *cir1* plants expressed *PR-1, PR-2, PR-5, GST1* and *PDF1.2* genes constitutively. Do *cir1* plants express additional SA-, ethylene- and jasmonate-responsive genes constitutively? In addition, *CIR1*-induced resistance to *P.parasitica* Noco2 appears to be independent of *PR-1* and *PDF1.2* expression. Which *CIR1*-induced genes are responsible for *P.parasitica* Noco2 resistance? Analysis of gene expression in *cir1* plants using techniques such as cDNA amplified fragment length polymorphism (cDNA-AFLP) (Durrant et al. 2000) or cDNA microarray analysis (Reymond et al. 2000, Schenk et al. 2000) should help to unravel *CIR1*-induced gene expression and the position of CIR1 in the SAR signal transduction network.

6.5. SAR as a mechanism to control plant disease

Further elucidation of the mechanisms underlying SAR could contribute to the exploitation of SAR in controlling plant diseases. One example of the successful application of SAR in controlling disease in agriculture is the development of the chemical plant activator Bion. The active compound of Bion is benzothiadiazole (BTH), which is a functional analogue of SA. In most plants studied, BTH activates the SAR pathway and is effective against a wide range of plant pathogens (Friedrich et al. 1996, Gorlach et al. 1996, Lawton et al. 1996). It has also recently been shown that Me-JA application reduced disease development of several necrotrophic plant

pathogens on Arabidopsis (Thomma et al. 2000). Over-expression of SAR regulatory proteins in transgenic plants is an alternative strategy to utilising SAR in plant disease control. It has been shown that transgenic Arabidopsis plants expressing the NPR1 protein are dramatically more resistant to the virulent pathogens *P.syringae* and *P.parasitica* (Cao et al. 1998). Recently, the observation that simultaneous activation of SAR and rhizobacteria-mediated ISR results in a significantly enhanced level of resistance to *P.syringae* (Van Wees et al. 2000) offers great potential for integrating both types of induced resistance in plant disease control.

It has been shown in Arabidopsis that the SA-dependent SAR pathway is more effective against certain pathogens (i.e. the biotrophic oomycete *P.parasitica*) whereas the jasmonate-dependent pathway is directed to other pathogens (i.e. the necrotrophic fungal pathogens *A.brassiciola* and *B. cinerea*) (Thomma et al. 1998). However, the recent characterisation of the *dnd1* mutant suggests SAR is more complex than signal transduction through two separate pathways. The *dnd1* mutant displayed the hallmarks of SA-dependent SAR (accumulation of SA, constitutive *PR-1* expression) (Yu et al. 1998) but was also resistant to *B.cinerea* (Govrin & Levine 2000). In addition, recent cDNA microarray analysis of over 2000 defence-related genes unexpectedly revealed that the expression of a large number of genes was co-ordinated by both SA and Me-JA (Schenk et al. 2000). A better understanding of plant resistance strategies is thus required. Furthermore, although Arabidopsis is a useful tool for dissecting SAR, it is likely that crop plants will show different SAR responses, both in comparison to Arabidopsis and to different pathogens. Map-based cloning of *CIR1*, the isolation of *CIR1*-homologues in crop plants and the analysis of pathogen resistance in *CIR1*-transgenic plants will thus be a useful goal.

Chapter Seven

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